GLUCOCORTICOID RECEPTOR MODULATION OF THE COLON CANCER MICROENVIRONMENT

Zuzanna Drebert

Promoter: Prof. Dr. Marc Bracke

Second promoter: Dr. Ilse Beck

Third promoter: Prof. Dr. Karolien De Bosscher

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SUPERVISORS

Promoter

Prof. Dr. Marc Bracke
Department of Radiation Oncology and Experimental Cancer Research
Ghent University

Second promoter

Dr. Ilse Beck
Department Health Sciences, Odisee University College
Department of Radiation Oncology and Experimental Cancer Research, Ghent University

Third promoter

Prof. Dr. Karolien De Bosscher
Department of Biochemistry
Ghent University

EXAMINATION COMMITTEE

Chairman of the Examination Committee

Prof. Dr. Bruno Verhasselt
Department of Clinical Chemistry, Microbiology and Immunology
Ghent University

Members of the Examination Committee

Prof. Dr. Karen Geboes
Department of Internal Medicine
Ghent University

Prof. Dr. Marleen Van Troys
Department of Biochemistry
Ghent University

Prof. Dr. Apr. Olivier De Wever
Department of Radiation Oncology and Experimental Cancer Research
Ghent University

Prof. Dr. Wim Vanden Berghe
Department of Biomedical Sciences
University of Antwerp

Prof. Dr. Frank Claessens
Department of Cellular and Molecular Medicine
University of Leuven

Front cover (from upper left to bottom right): histopathological section of chick chorioallantoic membrane invaded by HCT8/E11 cancer cells, human umbilical vein endothelial cells forming network of tube-like structures on Matrigel matrix (processed via Angiogenesis Analyzer), fluorescent visualization of glucocorticoid receptor localized predominantly in the nuclei of CT5.hTERT cells after glucocorticoid treatment, bioluminescent visualization of HCT8/E11luc+ cells via *in vivo* imaging system (IVIS).

Back cover: phase-contrast image of CT5.3hTERT cells

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SUMMARY

The tumor microenvironment comprises not only cancer cells but also stromal cells including cancer-associated fibroblasts (CAFs), endothelial cells, cells from the immune system and other cellular components, which together with the extracellular matrix form a complex network of interactions. The recognition of the role of tumor stroma in cancer progression provokes scientists to research these cells as targets and tools in cancer therapy.

Glucocorticoids, steroidal drugs with strong anti-inflammatory properties, are often used during chemotherapy of solid tumors, mainly to alleviate side effects of the cytotoxic treatment and to relieve cancer-associated pain. Activation of the glucocorticoid receptor via biding glucocorticoids, leads to suppression or stimulation of specific gene expression in mechanisms called transrepression and transactivation, respectively. Also other mechanisms of the glucocorticoid receptor are known. Although glucocorticoids are commonly used, their impact on solid tumor biology is controversial and not completely understood. Moreover, prolonged use of steroidal drugs is associated with harmful side effects. Therefore, experimental drugs called selective glucocorticoid receptor modulators, which would hold beneficiary anti-inflammatory properties without triggering detrimental side effects, are under intensive examination.

The aim of this doctoral project was to establish the role in impact of glucocorticoids and a selective glucocorticoid regulator, compound A, on the colon cancer microenvironment, namely on CAFs and endothelial cells. Consequently, we aimed to investigate the impact of glucocorticoid-affected CAFs on their progression-promoting role in cancer cells and in angiogenesis.

In both CAFs and endothelial cells, the glucocorticoid dexamethasone was able to drive the glucocorticoid receptor into the nucleus, leading to the transrepression of particular pro-inflammatory genes in TNFα-stimulated cells. Compound A had an impaired ability to translocate the glucocorticoid receptor, and although its anti-inflammatory properties in CAFs were modest, its effects were more pronounced in endothelial cells. As expected, only dexamethasone, and not compound A, upregulated the glucocorticoid receptor transactivation-dependent GILZ expression. Neither dexamethasone, nor compound A affected CAF or endothelial cell viability. However, compound A delayed cell growth in both cell lines. Additionally, in CAFs, dexamethasone inhibited the expression of multiple factors involved in cancer progression and angiogenesis, including hepatocyte growth factor, urokinase plasminogen activator and matrix metalloproteinase 2. Yet, this effect was not detected for compound A.

Furthermore, we could show that such extensive changes in the CAF-derived secretome affected these cells' impact on other cellular populations from the cancer microenvironment, namely

endothelial and colon cancer cells. Conditioned medium from solvent- and dexamethasone-treated CAFs (CM^{CTRL} and CM^{DEX}, respectively) increased endothelial cell proliferation, but did not affect typical characteristics of these cells. Interestingly, only CM^{CTRL} accelerated endothelial cell migration, suggesting the presence of glucocorticoid-sensitive pro-migratory molecules in the CAF-derived conditioned medium. As expected, CM^{CTRL} stimulated growth and invasion of glucocorticoid receptor-deficient colon cancer cells. In contrast, the effects of CM^{DEX} were substantially less pronounced. In the *in vivo* chick chorioallantoic assay, the CAF and colon cancer cell co-culture-derived tumors treated with dexamethasone were significantly less invasive than the tumors treated with solvent.

Combined, in this doctoral dissertation we present the beneficial therapeutic roles of glucocorticoids in the colon cancer microenvironment and angiogenesis, and we reveal additional information about the function of the selective glucocorticoid receptor modulator, compound A.

SAMENVATTING

De kankermicro-omgeving bestaat niet alleen uit kankercellen, maar ook uit stromale cellen zoals myofibroblasten, endotheelcellen, cellen van het immuunsysteem en andere cellulaire componenten, die samen met de extracellulaire matrix een complex interactienetwerk vormen. Het erkennen van de rol van het tumor stroma in de ontwikkeling van kanker zorgt ervoor dat wetenschappers de stromale cellen onderzoeken als doelwit bij kankertherapie.

Glucocorticoïden, steroïdale geneesmiddelen met krachtige anti-inflammatoire eigenschappen, worden vaak bij chemotherapie van vaste tumoren gebruikt, voornamelijk om de bijwerkingen van cytotoxische behandelingen tegen te gaan, en om kanker geassocieerde pijn te verlichten. Wanneer glucocorticoïden hun receptor activeren, onderdrukken of versterken deze de expressie van verschillende doelwitmoleculen via de respectievelijke mechanismen transrepressie en transactivatie. De glucocorticoïd receptor gebruikt ook andere mechanismen. Hoewel glucocorticoïden frequent worden gebruikt, is hun invloed op de biologie van vaste tumoren controversieel en niet volledig begrepen. Bovendien wordt het langdurig gebruik van steroïde geneesmiddelen geassocieerd met schadelijke bijwerkingen. Daarom worden experimentele moleculen genaamd selectieve glucocorticoïd receptor modulatoren, die gunstige anti-inflammatoire eigenschappen zouden bezitten zonder nadelige bijwerkingen te veroorzaken, intensief onderzocht.

Het doel van dit doctoraatsproject was om de rol van glucocorticoïden en de selectieve glucocorticoïd receptor modulator, compound A, op de micro-omgeving van colonkanker te onderzoeken, met name op uit colonkanker afgeleide myofibroblasten en endotheelcellen. Bijgevolg wilden wij de effecten van door glucocorticoïden geaffecteerde myofibroblasten op hun bevorderende rol in kankercellen en angiogenese bestuderen.

In zowel myofibroblasten en endotheelcellen kon het glucocorticoïd dexamethason de glucocorticoïd receptor naar de nucleus transloceren, wat leidde tot de onderdrukking van bepaalde pro-inflammatoire genen in TNFα-gestimuleerde cellen. Compound A had een verminderd vermogen om de glucocorticoïd receptor te transloceren, en hoewel de anti-inflammatoire eigenschappen in myofibroblasten bescheiden waren, waren deze effecten meer uitgesproken in de endotheelcellen. Zoals verwacht stimuleerde enkel dexamethason, en niet compound A, de glucocorticoïd receptor transactivatie-afhankelijke GILZ expressie. Dexamethason noch compound A hadden een invloed op de levensvatbaarheid van de myofibroblasten of endotheliale cellen. Echter, compound A vertraagde de celgroei van beide cellijnen. Bovendien remde dexamethason in myofibroblasten de expressie van verschillende factoren af die betrokken zijn bij de progressie van kanker en angiogenese, waaronder hepatocyt

groeifactor, urokinase plasminogeenactivator en matrix metalloproteinase 2. Dit effect werd echter niet terug gevonden bij compound A.

Bovendien hebben we laten zien dat zulke grote veranderingen in het myofibroblastische secretoom een invloed hadden op de impact die deze cellen teweegbrengen op andere celpopulaties van de kankermicro-omgeving, namelijk de endotheel- en colonkankercellen. Geconditioneerd medium van solvent- en dexamethason-behandelde myofibroblasten (respectievelijk CM^{CTRL} en CM^{DEX}) verhoogde namelijk de proliferatie van endotheelcellen, maar had geen invloed op de typische kenmerken van deze cellen. Het is interessant dat enkel CM^{CTRL} de migratie van endotheelcellen versnelde, hetgeen duidt op de aanwezigheid van glucocorticoïd-gevoelige pro-migratoire moleculen in het geconditioneerd medium van myofibroblasten. Zoals verwacht, stimuleerde CM^{CTRL} de groei en invasie van glucocorticoïd receptor-deficiënte colonkankercellen. De effecten van CM^{DEX} daarentegen, waren aanzienlijk minder uitgesproken. In de *in vivo* kuiken chorioallantoïsch analyse waren tumoren, ontstaan uit co-culturen van myofibroblasten en colonkankercellen, minder compact en significant minder invasief bij behandeling met dexamethason.

Samengevat, in dit proefschrift presenteren wij de positieve therapeutische rol van glucocorticoïden op de colonkankermicro-omgeving en op angiogenese, en onthullen we nieuwe informatie over de functie van de selectieve glucocorticoïd receptor modulator, compound A.

PART I: GENERAL INTRODUCTION

1. Cancer

Cancer has accompanied humans and animals throughout the history. The oldest information on this disease was found in an ancient manuscript dated back to 3000 BC and later evidence was uncovered in mummified bodies from Egypt and Ancient Nubia (Figure 1) [1-3]. Despite an immense progress in cancer-related research and largely evolved therapeutic and diagnostic methods in the past decades, it is estimated that only in 2012 cancer harvested 8.2 million lives worldwide [4]. Cancer can affect any part of the body, resulting in more than 100 types of malignancies [5], with lung and breast cancer appearing most common in men and women, respectively [4]. Only approximately 5-10% of all cancers are caused by inherited gene defects, as most of these cancers develop due to lifestyle-associated factors. Tobacco use, accounting for about 20% cancer deaths, belongs to the most important risk factors. Other factors include infections, unhealthy dietary habits, obesity, alcohol consumption, lack of physical activity, air pollution, and radiation [6,7].

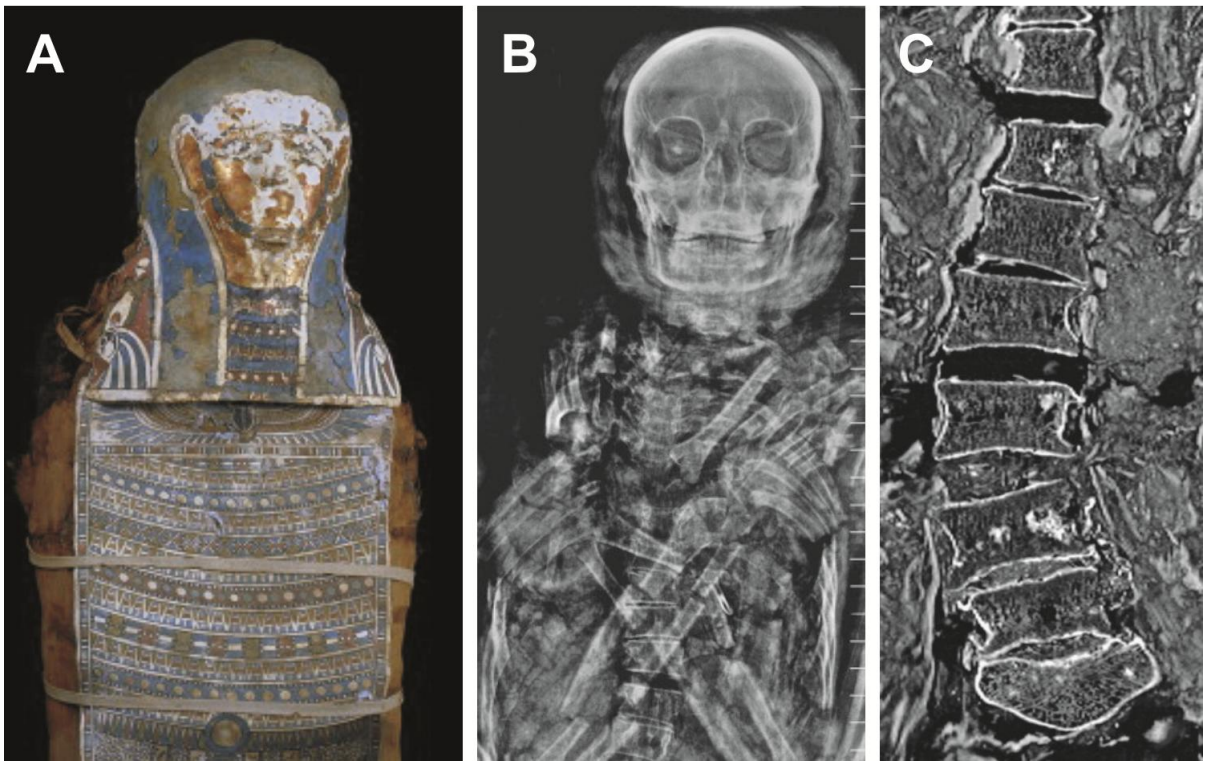

Figure 1. Example of an ancient incident of cancer. A) A 2250-year-old human mummy from the Egyptian Collection of *Museu Nacional de Arqueologia* in Lisbon B) X-ray digital image of the upper body C) A high-resolution multi detector computerized tomography scan of the lumbar spine region showing bone lesions associated with metastatic prostate cancer. Image adapted from [3].

1.1. Hallmarks of cancer

Not every abnormality in a tissue leads to cancer. However, some of the changes, if neglected, may increase the risk. Hyperplasia is a state in which cells proliferate at a higher rate than normally, but their function and morphology is not affected. In dysplasia, beside the faster division, cells have changed morphology and the tissue structure is disorganized. Carcinoma *in situ* is a more advanced form of dysplasia, and it might be a stage prior cancer, i.e. before abnormal cells invade the nearby tissues. Benign tumors do not spread to other tissues and after surgery usually do not grow back. Malignant tumors, in contrast, are aggressive, able to invade the surrounding tissues and form secondary (metastatic) tumors [5].

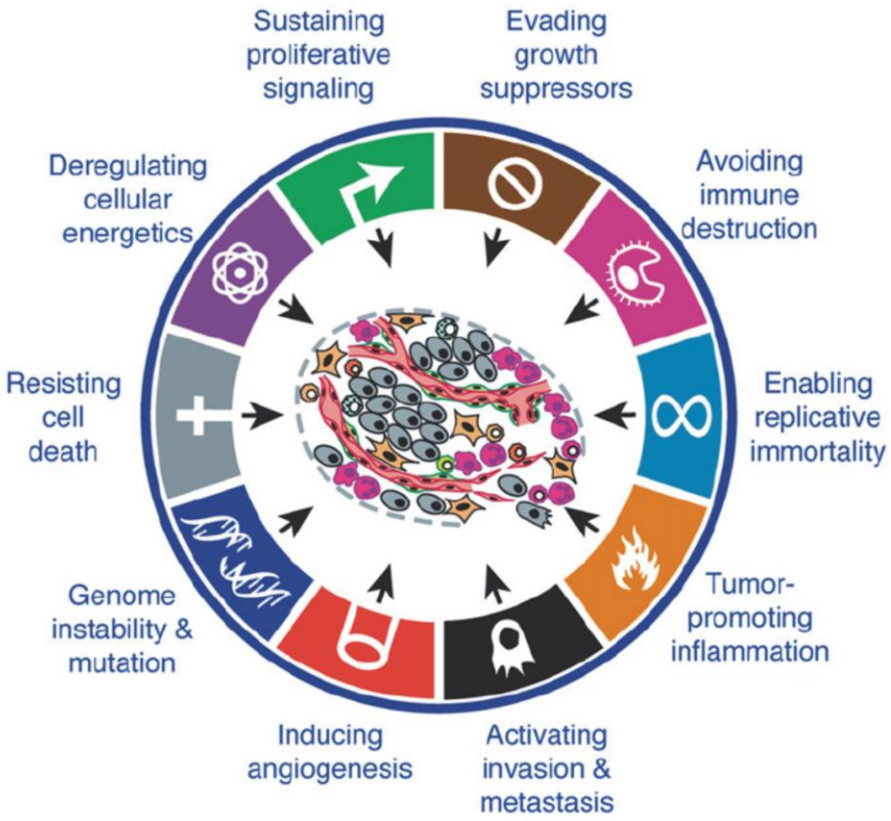

Figure 2. Hallmarks of cancer. Description in the text (section 1.1). Figure adapted from [8].

Carcinogenesis occurs when normal cells transform into cancer cells, which is characterized by genetic and epigenetic changes. During the carcinogenesis cancer cells acquire different properties, classified as hallmarks of cancer (Figure 2) [8]. Probably the most recognized characteristic of a cancer cell is its ability to sustain constant proliferation, which can be obtained via various strategies. In order to stimulate their growth, cancer cells can produce growth factors and receptors themselves, but also stimulate the surrounding non-cancerous cells to send growth signals. Moreover, even in the absence of growth factors cancer cells are able to activate

particular signaling pathways [8]. Proto-oncogenes and oncogenes (genes coding for transcription factors, signal transducers, growth factors and their receptors) play an important role during the carcinogenesis. Due to genetic mutations and extensive expression, oncogenes' products have a significant contribution in the uncontrolled cell division [9]. Next to the chronic cell proliferation, the replicative immortality guarantees the appearance of a new generation of cells, what in a physiological situation is controlled by cell senescence and subsequent cell death. Moreover, cancer cells acquire resistance to growth suppressors, factors from the outside and inside of the cell that normally negatively control cell division [8]. One of the best known tumor suppressors, tumor protein 53 (p53), is also called "the guardian of the genome", owing to its role in controlling the stability of the DNA, induction of cell cycle arrest, senescence and cell death. Therefore, inactivation of p53, which occurs mostly due to mutations in its own gene, marks one of the milestones in the process of tumorigenesis [10,11]. Unresponsiveness to growth suppressors leads eventually to a more drastic strategy of protecting the organism against damaged, infected or abnormally behaving cells, namely to programmed cell death (apoptosis), the natural destiny of every cell. Cancer cells, however, are able to resist apoptosis via sabotaging the apoptotic machinery, which can occur at multiple different levels, i.e. by increasing expression of anti-apoptotic factors or decreasing pro-apoptotic regulators [8]. A growing tumor needs an adequate supply of oxygen and nutrients, therefore, another hallmark of cancer is the ability of cancer to induce angiogenesis, a process of creating new vasculature from the existing network [12]. Tumor vasculature is chaotic and leaky, and as such contributes to the dissemination of cancer cells. In order for malignant cells to spread within the body, cancer cells induce the invasion-metastasis cascade which comprises multiple steps: (I) local invasion of neighboring tissue, (II) further cell migration through blood or lymphatic vessel walls (intravasation), (III) survival in circulation, (IV) escape from the circulatory system (extravasation) and (V) colonization of a new niche, which leads to the formation of metastases [8,13]. Yet, early dissemination of metastatic cells without primary tumor is recently increasingly documented [14,15]. A process called epithelial-to-mesenchymal transition (EMT) broadly regulates the initial steps of cancer cell invasion. After cells undergo EMT, they become mobile, lose their epithelial characteristics in favor of mesenchymal features, which leads to loosening of the tight connections with other cells and with the extracellular matrix (ECM) [16]. Moreover, recent research progress provided two additional hallmarks with potential generality: reprogramming of cell energy metabolism and escaping immune destruction by cancer cells. Furthermore, the complexity of the tumor microenvironment gained increased interest, since cells from the cancer's neighborhood, either recruited or attracted by cancer, such as macrophages, myofibroblasts, and endothelial cells, play a key role in cancer progression and protection via a constant exchange of signals [8].

1.2. Colorectal cancer

Carcinoma, cancer derived from epithelial cells, is the most common type of cancer. The majority of the colorectal cancers (CRCs) belong to the class of adenocarcinomas, i.e. derived from epithelium producing fluids or mucus [5]. In men, CRC is the third- and in women the second-most common cancer worldwide, with about 1.4 million cases and nearly 700,000 deaths reported in 2012 [4]. In the majority of cases, CRC arises sporadically, with risk factors, such as increasing age, the occurrence of colonic polyps, and environmental factors, such as red meat consumption, high-fat diet, smoking, sedentary lifestyle, obesity, and alcohol consumption [17]. Moreover, with the chronic inflammation being an additional important risk factor, individuals suffering from the inflammatory bowel disease, have a significantly higher chance of developing CRC [18]. Lynch syndrome (hereditary non-polyposis CRC) and the less frequent familial adenomatous polyposis (FAP) belong to hereditary syndromes associated with CRC and in total account for about 6% of cases [19]. Moreover, a family history of CRC is also an important risk factor, even despite the lack of evidence of any known inherited syndromes [20].

1.2.1 Mechanisms involved in molecular pathogenesis of CRC

Currently, there are two sequences identified that drive the transformation of normal colonic glandular epithelium to CRC. In both sequences transformation in the epithelium starts with the occurrence of aberrant crypt foci, followed by the formation of polyps, which, in turn, can progress into cancer. The traditional sequence involves the development of tubular adenomatous polyps which can evolve into adenocarcinomas. In the alternative sequence, the occurrence of so called sessile serrated polyps leads to progression to serrated CRC [21].

1.2.1.1. Genomic and epigenetic instabilities in CRC

Genomic and epigenetic instabilities are fundamental features of CRC that can drive the transformation of normal colonic epithelium to an invasive cancer. Three major phenotypes have been described to lead to CRC tumorigenesis: the chromosomal instability phenotype (CIN), the mutator-phenotype/DNA mismatch repair phenotype (microsatellite instability, MSI) and the hypermethylation phenotype hyperplastic/serrated polyp phenotype (the CpG island methylator phenotype, CIMP). Other mechanisms including involvement of microRNAs and inflammatory pathways have also been described [22].

CIN which can be recognized by multiple aberrations in the chromosomal structure, as well as in an abnormal number of chromosomes, forms the most common genomic instability and is characteristic for FAP inherited syndrome and the majority of sporadic CRCs. MSI is implicated in the Lynch syndrome, as well as a number of sporadic CRCs and the underlying mechanism involves an inactivation of DNA mismatch repair (MMR) genes [23]. Hypermethylation of CpG

island-rich gene promoters and global DNA hypomethylation are common epigenetic instabilities occurring in CRC. CRC containing exceptionally numerous incorrectly methylated CpG loci are classified as CIMP. Aberrant CpG island methylation can result in the inactivation of transcription of certain genes involved in cell cycle regulation. Genomic instabilities and epigenetic alterations lead to an accumulation of gene mutations and a deregulation of signaling pathways [22,23]. Figure 3 depicts the mechanisms involved in CRC tumorigenesis.

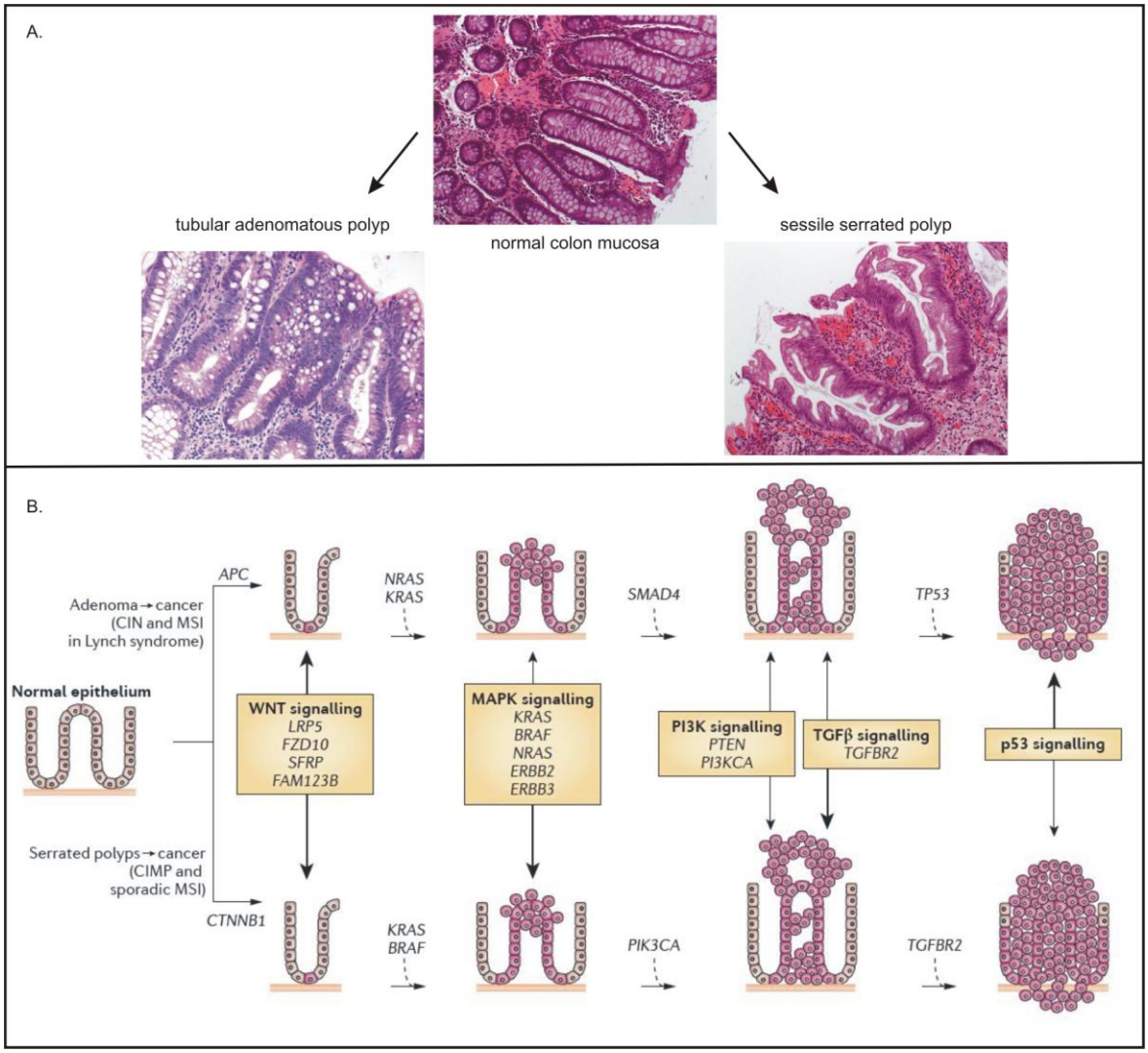

Figure 3. Sequences involved in colorectal cancer tumorigenesis. Two major sequences are recognized (adenomatous polyps to cancer and serrated polyps to cancer). (A) Histologic appearance of normal colon mucosa, adenomatous polyps and serrated polyps. Presense of sawtooth architecture of the crypts is characteristic for the serrated polyps. (B) The molecular networks affected in the particular stages are indicated within the frames. Both sequences share some genetic mutations (dotted lines), but they are also characterized by specific mutations. Figure adapted from [24-26] and [21].

1.2.1.2. Gene mutations and signaling pathways involved in CRC

The most common mutation occurring in sporadic CRCs is also associated with CIN and FAP. This mutation occurs in the adenomatous polyposis coli (*APC*) gene, which is a tumor suppressor factor

that negatively regulates the Wnt signaling cascade. A highly conserved Wnt pathway plays a key role in embryo growth and development, as well as in tissue homeostasis. In the activated Wnt pathway β-catenin is translocated from the cytoplasm to the nucleus, where it acts as a transcriptional co-activator to stimulate expression of Wnt target genes, such as genes encoding for Tcf-1, LEF1, c-myc, and Cyclin D [27]. When the Wnt pathway is inactive β-catenin is restrained in a protein complex, which includes APC. This leads to a proteasomal degradation of β-catenin. The *APC* gene mutation causes the synthesis of a truncated protein, unable to drive cellular β-catenin degradation. Inactivation of APC results in a constant activation of the Wnt cascade, leading to the uncontrolled stimulation of cell proliferation, differentiation, and migration. Wnt pathway can in certain cases also be affected at the β-catenin levels, independently of the APC functionality. Due to a mutation in β-catenin's *CTNNB1* gene, a wild-type APC protein is unable to degrade mutated β-catenin, subsequently leading to a similar outcome as with the APC gene mutation [22,23,28].

RAS is a family of oncogenes of which KRAS and NRAS play an important role in CRC. An activating mutation in the *KRAS* gene occurs in about 30% of all cancers and in approximately 40% of CRC cases [29]. *KRAS* encodes for a membrane-anchored guanosine triphosphate (GTP)- guanosine diphosphate (GDP)-binding protein (also known as p21) which acts as a downstream effector of the epidermal growth factor receptor (EGFR) to transduce growth stimulatory and pro-survival signals via B-Raf serine-threonine kinase and subsequent mitogen-activated protein kinase (MAPK) signaling. Mutations in *KRAS* lead to the constant activation of the pathway, resulting in enhanced cell proliferation and the possibility to escape apoptosis, disregarding EGFR activity. Therefore, patients with the mutated *KRAS* and also *NRAS* are not eligible for anti-EGFR therapy [30,31]. Moreover, mutations in the *BRAF* gene, occurring in 10% of CRC cases, can lead to a similar effect. The most common mutation in *BRAF* is a substitution of the amino acid valine to glutamic acid (V600E mutation), leading to the constitutive activation of B-raf protein [32]. Transformation of the epithelium to serrated polyps is attributed to *BRAF* mutations, which are frequent in MSI and highly implicated in CIMP CRCs [23].

Mutations in genes encoding for elements of the phosphoinositide-3 kinase (PI3K) signaling pathway are observed in about 40% of CRCs. The PI3K pathway can be activated by four types of sensors: receptor-tyrosine kinases (RTKs, such as EGFR or insulin-like growth factor 1 receptor, IGF1R), integrins (proteins responsible for cell-extracellular matrix connections) and the cytokine- or G-protein-coupled receptors. The PI3K/Akt/mammalian target of rapamycin (mTOR) pathway leads to the activation of multiple transcription factors that regulate cell cycle, subsequent cell proliferation, and survival. PI3K is the kinase that phosphorylates and activates Akt (protein kinase B, PKB) using the phosphatidylinositol-3,4,5-trisphosphate (PIP3) as a substrate. Mutations in

PIK3CA gene encoding for PI3K, can lead to the enhanced activity of the pathway and a subsequent increase in pro-survival signals. Moreover, mutations in the *PTEN* gene, encoding for the tumor suppressor that negatively regulates the levels of PIP3, often occur in CRC, leading to an uncontrolled activation of PI3K/Akt/mTOR signaling [33].

The transforming growth factor (TGF)β signaling pathway, a tumor suppressing cascade, is often distorted in CRC. Mutations, common in most MSI CRCs, leading to the inactivation of the TGFβ cascade, can occur in TGFβ receptor genes (*TGFBR1* and *TGFBR2*), in the downstream effector genes (*SMAD2*, *SMAD4*), and in genes encoding for members of the TGFβ superfamily (*ACVR2*). However, an excessive TGFβ expression in CRC (and several other types of cancer) correlates with tumor progression. TGFβ when secreted by cancer cells has a strong influence on the tumor microenvironment – it suppresses the inflammation, promotes angiogenesis and recruits myofibroblasts, known to support cancer progression [34,35].

p53 reduces the risk of the malignant transformation by inducing apoptosis in cells that suffer from cellular stress, such as hypoxia, DNA damage or oncogenic activation. In such circumstances, p53 acts as a transcription factor to trigger the expression of pro-apoptotic molecules (e.g. Bax, Noxa, PUMA) and to inhibit expression of pro-survival factors (e.g. Bcl-2, Bcl-xL). Loss of functional p53 leads to an uncontrolled cell proliferation and evasion from the cell death. Mutations in the *TP53* gene occur in late stages of about 50% of CRCs cases and the presence of the aberrant p53 is associated with CRC aggressiveness and metastasis [36,37].

1.2.2. Therapy and prevention of colorectal cancer

Most of the signs and symptoms of CRC are non-specific and besides that, the early stage CRC can be asymptomatic. The endoscopic examination of the large intestine, colonoscopy, is the most universal and accurate test for CRC screening. Besides the visual evaluation, it provides opportunity to obtain biopsies and to remove polyps. The obtained tissue is further histopathologically examined. Independently, the disease extent can be evaluated by an imaging test, such as CT scan of the abdomen, chest and pelvis [38]. The stage of the tumor is assessed according to the Tumor Node Metastases (TNM) staging system. The “T” category represents the primary tumor, the “N” describes whether the cancer is spread to adjacent lymph nodes and the “M” category specifies whether the tumor is metastatic. When the categories are ascribed, the overall stage of the tumor is determined (extending from stage 0 to IV) [39].

Surgical resection forms the only curative method for localized CRC, i.e. a tumor that is localized at the colon wall and/or the nearby lymphatic nodes. Preoperative (neoadjuvant) radio- or chemoradiotherapy is a common strategy for the treatment of locally advanced rectal cancer, invading into neighboring organs. In order to remove potential micrometastases and to subsequently reduce the recurrence of the disease, the postoperative (adjuvant) chemotherapy is

the next major step in CRC therapy. Chemotherapy is planned individually for each patient and in most cases comprises a combination of several drugs. Chemotherapy is associated with several side effects, such as emesis, diarrhea, mucositis, hair loss, fatigue, cardiotoxicity and other toxicities [21].

1.2.2.1. Cytotoxic chemotherapy

Developed in 1957 by Charles Heidelberger and colleagues, 5-fluorouracil (5-FU) was the first chemotherapeutic used for the treatment of CRC [40]. Metabolized 5-FU acts as an inhibitor of thymidilate synthase, irreversibly blocking synthesis of thymidine, a nucleoside necessary for the DNA replication [41]. Later on, the addition of leucovorin (folinic acid) was proven to enhance the effectiveness of 5-FU. Nowadays, the first-line treatment for metastatic CRC is most often a combination of oxaliplatin (platinum derivative) with 5-FU or with an oral formulation of 5-FU capecitabine (FOLFOX or CAPOX regimen). Another approach is a combination of irinotecan (topoisomerase I inhibitor) with 5-FU (FOLFIRI regimen). A triplet regimen with use of oxaliplatin, 5-FU and irinotecan (FOLFOXIRI) has also shown to be effective [21,41].

1.2.2.2. Targeted therapy

Chemotherapy regimens are usually enriched by the use of targeted therapy agents. In CRC treatment these agents include monoclonal antibodies (mAbs) against EGFR or against vascular endothelial growth factor (VEGF)-A, a soluble protein that stimulates creation of new blood vessels (Figure 4) [21]. The majority of CRCs (over)express EGFR, which correlates with poor prognosis. The use of mAbs such as cetuximab and panitumumab showed efficacy in chemotherapy-naïve patients, as well as in individuals with emerging cytotoxic chemotherapy resistance. The use of EGFR mAbs, however, is only beneficial for patients who do not carry activating mutations in the *KRAS* and *NRAS* genes (both in exons 2, 3, and 4) detected in more than 40% of patients, and as such the *RAS* mutational status serves as a negative predictive biomarker for the anti-EGFR therapy [30,31,42]. Growing tumor needs additional oxygen and nutrients supply [43]. Subsequently, endothelial cells (ECs) are attracted to the hypoxic regions and stimulated by VEGF to create a new vascular network. Therefore, targeting the angiogenesis forms an important strategy in the anti-cancer therapies [44]. When released by cancer cells or adjacent stromal cells VEGF binds to its receptor (VEGFR) leading to an increased proliferation, migration of ECs and the increased vessel permeability. Bevacizumab, a mAb developed against VEGF-A, applied in the combination with a FOLFIRI or a FOLFOX regimen has demonstrated an improved median of progression-free survival in patients with metastatic CRC. Another anti-angiogenic drug used together with chemotherapy is aflibercept, a recombinant fusion protein that contains a VEGF-binding domain (recombined from VEGFR), and therefore, it acts as a VEGF-trap [42,45]. Moreover, in 2013 a novel chemotherapy agent for refractory metastatic CRC was

approved. Regorafenib, a tyrosine kinase inhibitor able to block several receptors (VEGFR-1, -2, -3, TIE-2 and others) demonstrated a significant clinical benefit [46].

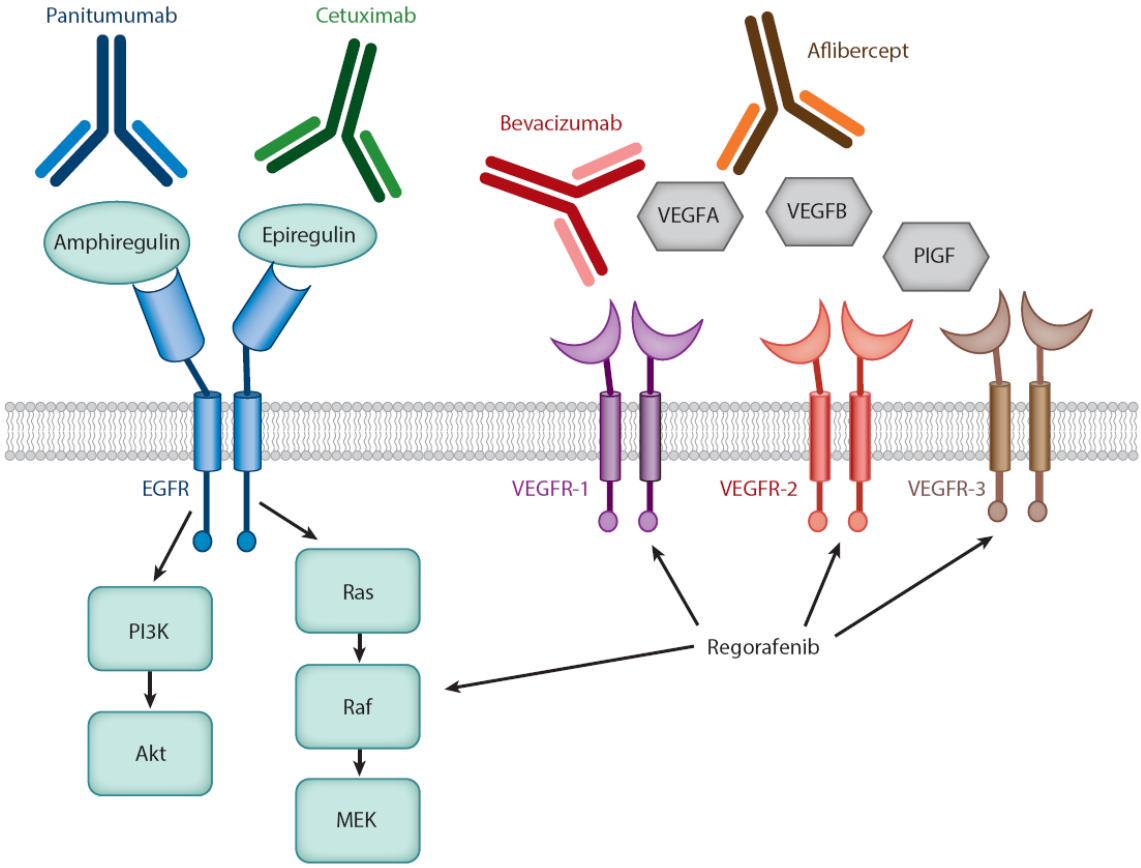

Figure 4. Therapeutics targeting signaling pathways in CRC. Description in the text (section 1.2.2.2.) Figure adapted from [42].

1.2.2.3. New therapies

Immunotherapy, which uses the patient's immune system to fight the disease, is a new promising approach in cancer treatment [5]. After obtaining positive results in other types of cancer, use of immunotherapy, including nivolumab, an antibody developed against programmed cell death (PD)-1 protein, was shown effective in CRC patients with high-level MSI [47]. PD-1 is a receptor that contributes to suppression of inflammatory activity of T-cells and subsequently induces tolerance. PD-1 is therefore an immune checkpoint against autoimmunity, leading to increased apoptosis of antigen-specific T-cells and reduced apoptosis of regulatory T-cells. Inactivation of PD-1 in T-cells stimulates them to attack cancer cells [48].

Cancer stem cells (CSCs) form a subset of cancer cells that share characteristics with normal stem cells, giving them the ability of self-renewal and differentiation. Therefore, CSCs are believed to be responsible for metastasis formation and therapy resistance [49]. BBI608 is a stem cell inhibitor that targets several pathways associated with tumorigenesis [50]. This small molecule was shown to inhibit gene transcription driven by STAT3, an important factor to maintain cancer

stemness, as it regulates expression of genes implicated in cell survival, self-renewal, invasion and immunosuppression [51]. BBI608 decreased in a dose-dependent manner protein levels of Sox-2, Nanog, survivin and c-Myc, being down-stream factors of STAT3 [52] and levels of β-catenin, contributing to the deactivation of the Wnt cascade [27]. Subsequently, in cancer stem cells obtained from various cancer types, BBI608 was able to block spherogenesis and had a cytotoxic effect on cells with high stemness potential. Furthermore, BBI608 blocked cancer relapse and metastasis *in vivo* [50]. Since the phase I studies showed promising results, Boston Biomedicals, Inc. conducts phase II clinical trial for the use of BBI608 in the combination with panitumumab, capecitabine and cetuximab in adult patients with advanced CRC (clinicaltrails.gov identifier: NCT01776307).

Patients with KRAS and NRAS mutation cannot benefit from the anti-EGFR therapy [30]. Reolysin®, an oncolytic virus designed by Oncolytics Biotech Inc., is a formulation of a human reovirus strain that targets cancer cells with an activated RAS pathway, and meanwhile has very little effect on cells in which RAS signaling is inactive [53,54]. The KRAS mutation is also common for melanomas and the intravenous application of Reolysin had a promising outcome in phase II clinical trial performed in metastatic melanoma patients [55].

1.2.2.4. Repurposed drugs

Drug repurposing (reprofiling, repositioning) indicates application of well-known and well-characterized drugs to treat new diseases [56]. A ReDO (Repurposing Drugs in Oncology) project focuses on known non-cancer drugs and their potential use in cancer therapy and prevention [57]. Recent accumulating evidence shows that non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin and diclofenac, are effective in preventing premalignant polyps formation and the development of CRC [58,59]. The use of aspirin reduced the CRC risk by 24% and mortality by 35% [60], and it was especially effective in individuals with Lynch syndrome [61]. Moreover, a regular aspirin use after CRC diagnosis reduced the disease recurrence in patients with a *PIK3CA* gene mutation [62]. Nevertheless, the mechanism behind the beneficial effects of aspirin is unclear. Possible actions include an inhibition of the nuclear factor (NF)κB and Wnt pathways (being also downstream the PI3K), as well as its modulating effects through the tumor microenvironment [42,63].

An anti-diabetic drug metformin, the most commonly prescribed medicine for diabetes type 2, is another potential repurposed drug [64]. Metformin treatment is associated with substantially lower risk of colorectal cancer in patients with type 2 diabetes, but it also decreased the risk of occurrence in other malignancies including pancreas, hepatocellular, and prostate carcinomas [64-66]. Molecular action of metformin in cancer cells involves mainly the inhibition of mTOR signaling pathway, playing an important role in cell proliferation. Moreover, metformin-mediated

reduction of insulin and IGF-1 levels is also suggested as an anti-cancer mechanism [66]. Furthermore, metformin was shown to induce 5-FU- and oxaliplatin-driven cell death in chemotherapy resistant colon cancer cells *in vitro*, which was associated with downregulation of the Wnt pathway [67].

Other non-cancer drug candidates of which there is pre-clinical and clinical evidence of chemopreventive or anti-cancer actions include cimetidine - commonly used to treat stomach ulcers, nitroglycerin - used for high blood pressure and heart failure, and mebendazole - used against parasitic worm infestations [57].

1.3. Cancer microenvironment

Tumors are no longer recognized as a homogenic mass of cancer cells. Past decades of research have resulted in the recognition of cancer stem cells (CSCs). CSCs located within the malignant cell population play a key role in driving cancer growth [49]. Additionally, different cellular and non-cellular components of the tumor microenvironment are crucial for cancer development. The tumor mass comprises non-cancerous cells, such as myofibroblasts, recruited at the invasive front of the tumor, endothelial cells (EC) that together with pericytes are structural elements of the tumor vasculature, cells of the immune system, and other cellular components characteristic for certain types of cancer (such as adipocytes in breast cancer). Moreover, the extracellular matrix (ECM) forms an active scaffold for the cross-talk between particular cellular populations. The dynamics occurring within the tumor microenvironment share similarities with the processes of wound healing and inflammation, therefore a tumor was once described as a "wound that does not heal" [68,69]. Figure 5 depicts a simplified model of a tumor microenvironment.

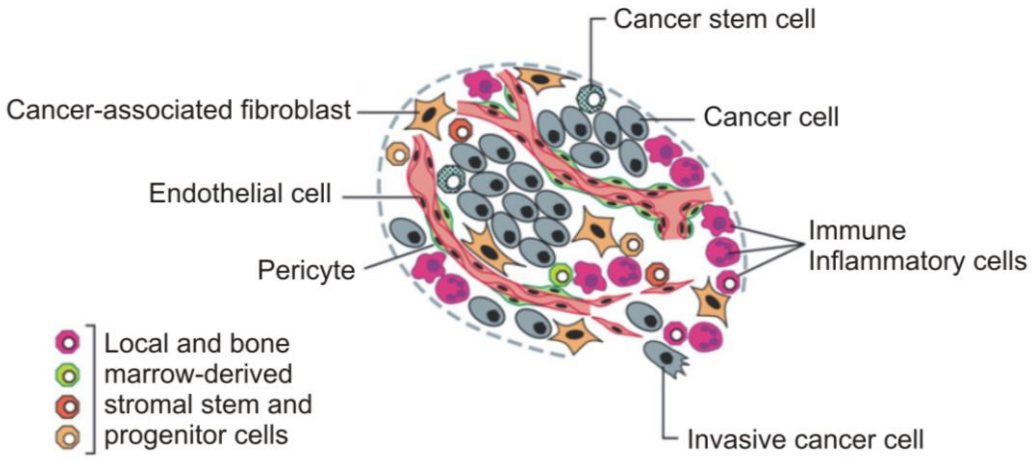

Figure 5. Model of tumor microenvironment. Figure adapted from [8].

1.3.1. The extracellular matrix

The ECM is a dynamic network of macromolecules (proteoglycans and fibrous proteins), which are secreted by the resident cells. The ECM acts as a biochemical and structural support for cellular components of the tissue, it contributes to cell adhesion, polarity, migration, differentiation, proliferation and cell-to-cell communication. The ECM forms a reservoir for growth factors that are embedded within it and certain ECM components may act as signal co-receptors or presenters by binding to specific growth factors. Each type of mammalian connective tissue has a different composition of its ECM that contributes to its particular tissue function, however, the ECM can be divided into two main types: the basement membrane and the interstitial (stromal) matrix. The basement membrane is a compact, laminin-, nidogen-, and collagen type IV-rich ECM, produced

by epithelial, endothelial and stromal cells, and acts as a barrier between stroma and epithelium or endothelium. The interstitial matrix, in contrast, is less compact, more hydrated and rich in fibrillar collagens, proteoglycans and polysaccharide gels. The ECM is highly plastic and its remodeling is strictly regulated during tissue development, thus the abnormal ECM dynamics are characteristic for many diseases, including cancer. In epithelial cancers the dissolution of the basement membrane is a crucial step in cancer invasion. Moreover, tumors are significantly stiffer than surrounding healthy tissues as a result of its particular ECM deposition and remodeling by fibroblasts and myofibroblasts recruited by cancer cells [70-73]. Figure 6 depicts examples of common macromolecules present in an ECM.

1.3.2. Myofibroblasts

The presence of myofibroblasts is characteristic for the physiological processes during embryo development and wound healing. In response to injury, residential fibroblasts acquire some characteristics of smooth-muscle cells and differentiate to myofibroblasts that produce and secrete components of the wound's ECM. Owing to their contractile properties myofibroblasts play a major role in the maturation and contraction of the granulation tissue. Subsequently, myofibroblasts contribute to scar formation and remodeling of the granulation tissue, following a release of proteolytic enzymes (such as matrix metalloproteases, MMPs). Eventually, in healing tissues the number of myofibroblasts declines due to apoptosis [74,75]. Myofibroblasts, besides fibroblasts, can be recruited from various origins, i.e. from local or bone marrow-derived mesenchymal stem cells, endothelial cells, pericytes and other cell types undergoing EMT. Myofibroblasts have a polarized spindle-shaped morphotype and due to their contractile properties are highly motile. Additionally, myofibroblasts can be identified by the presence of typical markers, such as alpha-smooth muscle actin (αSMA), vimentin, podoplanin, desmin, neural-cadherin (N-cad) and the absence of certain cell type-specific markers, including cytokeratin, which is characteristic for epithelial cells [76]. Moreover, myofibroblasts produce and secrete various structural components of the ECM, such as collagens (type I, II, IV), fibronectin, tenascins and decorin [77,78]. During tumorigenesis myofibroblasts are recruited by cancer cells, and therefore, they are also known as cancer-associated fibroblasts (CAFs). Abundant in the tumor microenvironment, CAFs' presence is persistent in contrast to the physiological wound healing process. CAFs are activated mainly via TGFβ signaling, and in turn they support cancer progression via secretion of various cytokines, growth factors, chemokines and enzymes, such as CCL5 (RANTES), IL-6, hepatocyte growth factor (HGF), fibroblast growth factor (FGF), insulin-like growth factor-1 (IGF-1), MMPs, and tenascin C (TNC). CAF-released factors contribute to an increased cancer cell invasion, motility, survival, and also a stimulation of angiogenesis via the secretion of VEGF and other factors [76].

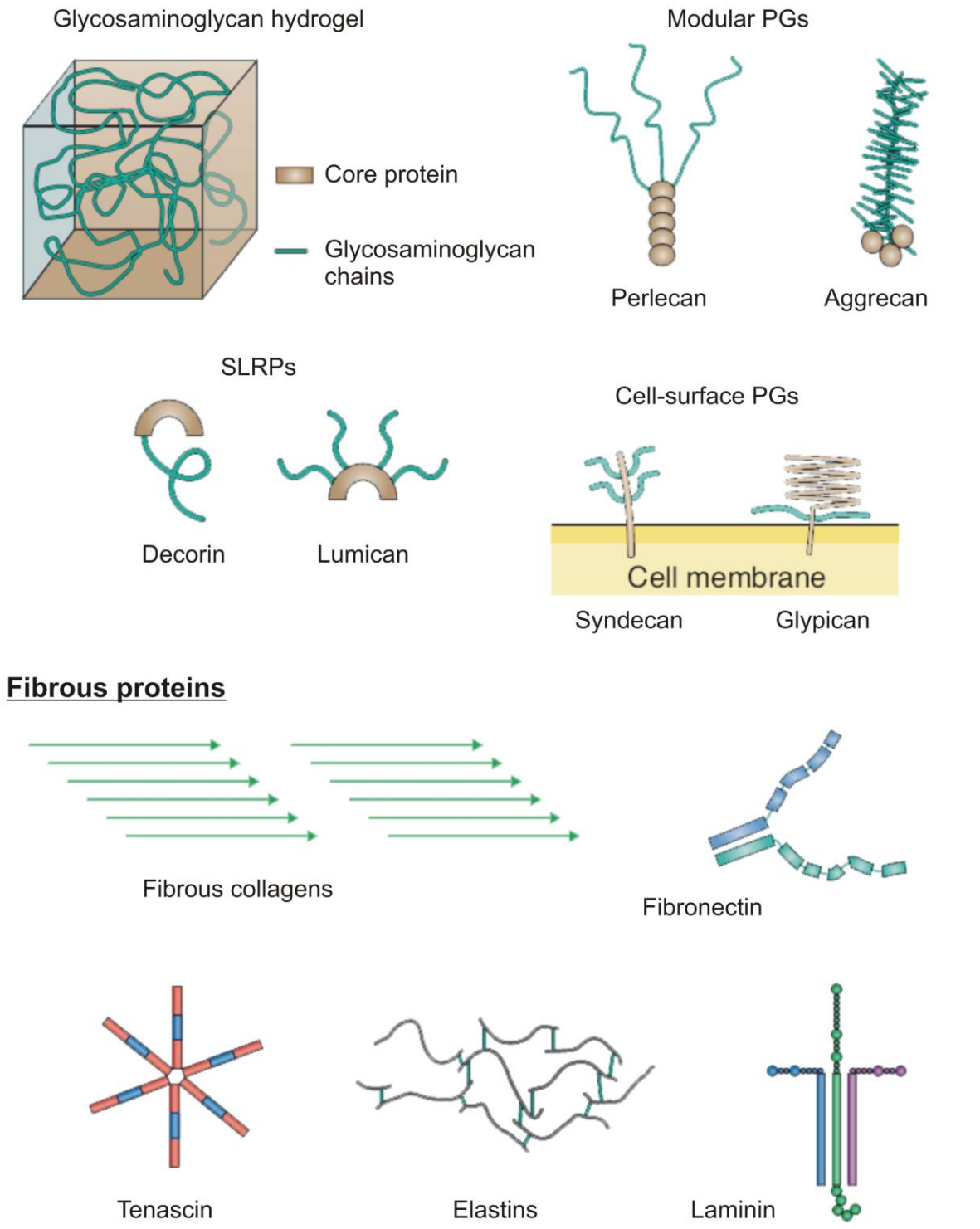

Figure 6. Extracellular matrix macromolecules. Proteoglycans (PGs) comprise of glycosaminoglycan (GAG) chain linked with a protein core. PGs are highly hydrophilic and involved in multiple signaling pathways, cell adhesion, migration and proliferation. PGs are classified into three families: SLRP – small leucin-rich PGs, modular PGs and cell-surface PGs. Collagen is the most common fibrous protein within the interstitial ECM and its main structural component. Fibronectin plays a key role in the cell adhesion by binding collagens and integrins. Elastins are highly elastic proteins that contribute to the tissue flexibility. Laminins are the main components of base membranes. Tenascins have cell adhesion-modulating properties. Figure adapted from [73].

1.3.3. Vascular endothelial cells and pericytes

Endothelial cells (ECs) are mesodermal components of the blood vessels inner surface. In vascular capillaries, ECs form a semi-selective monolayer that separates the blood from the surrounding tissue, guaranteeing at the same time an exchange of nutrients, waste products and gases. Perivascular stromal cells (pericytes) provide structural support to ECs [79]. Inadequate number of pericytes in the tumor vasculature causes vessel leakiness and is associated with a poor prognosis and metastasis in CRC [79,80]. Hypoxia (inadequate oxygen concentration) can occur in a quickly growing tumor mass [8,43]. In order to provide the necessary supply of nutrients and oxygen to the tumor, cancer cells and other cells from the tumor microenvironment (CAFs and tumor-associated macrophages) secrete factors to stimulate angiogenesis. ECs actively respond to vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and other pro-angiogenic factors. Because of a constant, uncontrolled growth factor flow, tumor vasculature greatly differs from the healthy situation. As such, tumor ECs are of irregular size and shape, affecting the endothelial monolayer's structure and function. Subsequently, formed vessels are fragile and leaky, show irregular diameters, and are chaotically organized, resulting altogether in an abnormal blood flow and a facilitated cancer cell intravasation [79]. The process of angiogenesis is further described in section 1.4.

1.3.4. Inflammatory cells

Various types of cells belonging to the immune system are attracted to the tumor site. T-lymphocytes are very abundant, making up to 10% of the cellular components of a tumor. Among the many populations of T-cells, CD8+CD45RO+ cells are capable of targeting and killing cancer cells. CD4+ helper 1 T-cells (Th1) produce IL-2 and interferon-γ and together with CD8+CD45RO+ are linked with a good prognosis. In contrast, Th2 and Th17 cells are associated with tumor growth, and furthermore, CD4+ T regulatory cells (Tregs) have tumor-promoting properties due to their immunosuppressive actions [68,81]. B-lymphocytes can be found at the invasive edge of the tumor and in tumor-adjacent lymph nodes. Their presence, however, similarly to natural killers (NK) and NKT cells, is linked with a good prognosis in some cancers [68]. Tumor-associated macrophages (TAMs) belong to "corrupted" cells that enhance cancer progression via stimulation of cancer cell migration, invasion and metastasis [82]. Because of their excessive production of growth factors and inflammatory cytokines, TAMs maintain a state of chronic inflammation within the tumor microenvironment. Furthermore, TAMs are attracted to the hypoxic areas of the tumor, and thanks to their VEGF secretion, they are important contributors of tumor angiogenesis. By releasing MMPs, TGFβ, EGF and other factors, TAMs also strongly influence ECM remodeling, cancer cell invasion and cell extravasation. Moreover, in pancreatic cancer macrophages were proven to activate myofibroblasts [82-84].

1.4. Angiogenesis

Angiogenesis is a process in which a new vascular network is created from the existing one [85]. This strictly regulated process due to the balance between pro- and anti-angiogenic endogenous factors (Table 1), is essential to tissue development, wound healing and during menstrual cycle.

Table 1. Examples of endogenous regulators of angiogenesis. Table adapted from [86] and [87]. *TGFβ has a controversial role in angiogenesis, which depends on the cancer type and cellular context [34].

Activators	Inhibitors
Growth Factors	
Angiogenin	TGFβ*
EGF	
FGFs	
HGF	
Platelet-derived growth factor (PDGF)	
TGFβ*	
Tumor necrosis factor (TNF)α	
VEGFs	
Cytokines	
IL-1	IL-10
IL-6	IL-12
IL-8	
Proteases and proteases inhibitors	
ADAM-17	ADAMTs
Cathepsin	PAI
MMP-2 (gelatinase A)	TIMP
MMP-3 (gelatinase B)	
MMP-9 (stromelysin-1)	
uPA	
Other endogenous regulators	
Angiopoietin-1	Angiopoietin-2
Endothelin	Angiostatin
Erythropoietin	Angiotensin
Hypoxia	Arresten
NO synthase	Canstatin
Prostaglandin E_2	Endostatin
$\alpha_5\beta_3$ integrin	Interferon-α
	Thrombospondin-1, -2
	Vasostatin

In a physiological situation, angiogenesis is a transient process, which is completely inhibited after a strict period of time. Disturbance of this balance can lead to a pathological insufficient angiogenesis (e.g. in the ischemic chronic wounds) or to an excessive angiogenesis (e.g. in cancer, ocular disorders and rheumatoid arthritis) [85,87]. As stated above, tumors cannot expand without nutrient and oxygen supply [43]. Cancer and cancer-associated cells secrete a plethora of pro-angiogenic factors, resulting in a constant distortion of the balance between activators and inhibitors of angiogenesis, and subsequently in an uncontrolled and chaotic formation of vasculature [86].

1.4.1. Stages of blood vessel sprouting

Angiogenesis occurs in several phases controlled by a complex net of regulation taking place in space and time. In a developing vessel, particular ECs acquire different roles, phenotypes and responses to the incoming signals. Sprout initiation occurs when one of the ECs responds to a pro-angiogenic signal by extending the filopodia. Such cell, called a tip cell, is still connected to the parental vessel while migrating outwards. At the same time the tip cell blocks filopodia formation in the neighboring cells. The tip cell continues to migrate toward the pro-angiogenic stimulus, while the neighboring ECs (stalk cells) follow it by migrating and proliferating, without disconnecting from the parental vessel. Subsequently, a new sprout forms a lumen to connect with another outgrowing sprout or vessel. Cell junctions with the ECs from the other sprout are established, and lastly, a complete loop is created. Eventually, the new structure is covered by pericytes and basement membrane, and such newly formed vessel can act as a parental vessel for a new outgrowing sprout [88].

1.4.2. Molecular mechanisms of angiogenesis

A multitude of factors were described as direct or indirect regulators of angiogenesis (Table 1). Particular molecules can be produced by various cell types as soluble factors or as membrane-linked proteins. Figure 7 depicts important signaling events occurring during tumor angiogenesis. Because of their role in cancer development many of these pathways became a target for cancer therapy [89].

1.4.2.1. Hypoxia-induced mechanism

Hypoxia is an important driver of angiogenesis and is counteracted by actions of the hypoxia inducible factor (HIF)-1. Under normoxia, HIF-1 is restrained from action due to hydroxylation of HIF-1 subunit α (HIF-1α), which is subsequently trapped in the E3 ligase protein complex, leading to its ubiquitination and degradation. Under hypoxic conditions, however, HIF-1α accumulates in the cytoplasm and dimerizes with the HIF-1β subunit to form a complete transcription factor that upon binding to the hypoxia-responsive elements (HRE) regulates expression of several target genes including VEGF, angiopoietins, PDGF-B, cytokines and ECM proteases [90,91]. Interestingly,

HIF-1's transcriptional activity can also be stimulated via PI3K/Akt or RAS/MAPK signaling cascades, which are often hyperactivated in cancer [92]. Pro-angiogenic factors, abundantly secreted by cancer cells, attract ECs and stimulate neovessel formation.

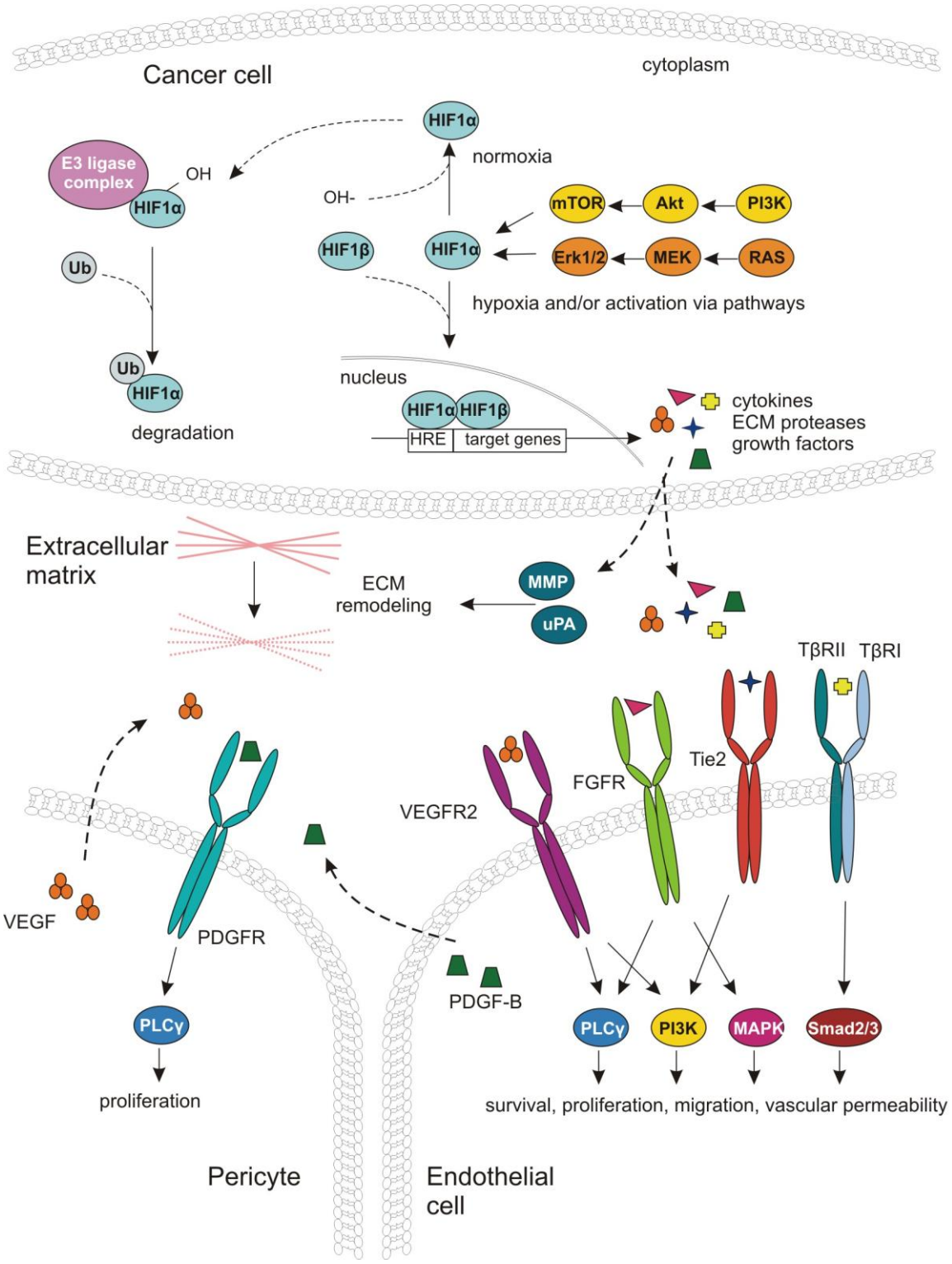

Figure 7. Schematic representation of selected molecular mechanisms involved in the tumor angiogenesis. Cancer cell produces factors to counteract the inadequate oxygen concentration. HIF-1 is a transcription factor responsible for activation of genes encoding for growth factors, cytokines and ECM proteases. Detailed description of the mechanisms is in section 1.4.2.

1.4.2.2. VEGF signaling

Vascular endothelial growth factor (VEGF) signaling has an essential role in angiogenesis. The VEGF protein family consist of several glycoproteins including the most studied VEGF-A, a factor implicated in vasculogenesis, angiogenesis, modulating vascular permeability, stimulation of migration, and proliferation of ECs. VEGF-B plays a crucial role in cardiac development, VEGF-C and VEGF-D are responsible for lymphatic vessel development. Endocrine gland (eg)VEGF targets the endocrine gland endothelium and the placental growth factor (PlGF), originally discovered in human placenta, was shown to play a role in modulating inflammation associated with pathological angiogenesis [45,93,94]. VEGFs are ligands for the tyrosine kinase receptors (RTKs) VEGFRs (mainly VEGFR-1, -2 and -3) expressed in ECs. VEGF-A binds and activates VEGFR-1 and VEGFR-2, however, most pro-angiogenic potential is generated via a VEGFR-2 activation. VEGFR-1 has a high affinity for VEGF-A, but its RTK activity is much weaker than VEGFR-2, therefore VEGFR-2 is proposed to act as a negative regulator of VEGF signaling, by acting as a VEGF-trap [95]. Dimerization and autophosphorylation of ligand-bound VEGFR-2 leads to the activation of several signaling pathways resulting in the increased EC proliferation, migration, and vascular permeability. These signaling pathways include phospholipase C (PLC)-γ/protein kinase (PK)C and subsequent MAPK and PKD signaling and also stimulation of PI3K signaling cascade via adaptor molecule Src homology 2 and β cell (Shb), that binds the phosphorylated VEGFR-2 [93].

1.4.2.3. Fibroblast growth factors and their receptors

Acidic and basic fibroblast growth factors (a- and bFGFs) are proven to be essential factors during the angiogenic response. FGFs produced by cancer or stromal cells bind to heparan sulfate proteoglycans (HSPGs), abundantly present on the surface of most cells and within the ECM. HSPGs-bound FGF forms a reservoir of the growth factor, facilitating its release in a controlled manner. Moreover, HSPGs serve as co-receptors to modulate FGF signaling. FGFs act as ligands for the FGF RTKs (FGFR-1, -2, -3 and -4). Upon binding to its ligand, an FGFR dimerizes and autophosphorylates leading to the activation of several signaling pathways including Ras/MAPK and PLC-γ/PKC, resulting in cell proliferation, migration and survival [96,97].

1.4.2.4. Angiopoietins and TIE receptors

Angiopoietin (ANGPT)-1 and -2 are ligands for the Tyrosine kinase with immunoglobulin-like and EGF-like domains (TIE) receptors, of which signaling is essential for maintaining vessel stability. Upon ANGPT-1 binding, TIE-2 undergoes autophosphorylation and subsequently activates PI3K/Akt cascade in ECs. The ANGPT-1 is associated with vessel maturation via enhanced EC migration, adhesion and survival. ANGPT-2, on the other hand, promotes vascular regression, by disrupting connections between ECs and perivascular cells, but in the presence of VEGF it

stimulates vessel sprouting [98]. Elevated levels of ANGPT-2 have been correlated to poor prognosis in several cancer types [99].

1.4.2.5. TGFβ and its receptors

As mentioned in section 1.2.1.2., aberrations in TGFβ signaling are common in CRC. TGFβ cytokines are released into the ECM in a latent dimerized complex. Activation of the protein can occur via proteolytic reaction catalyzed by plasmin or MMPs (such as MMP-2 and -9). TGFβ cytokines bind then to the serine/threonine kinase receptor type II (TβRII) which in turn phosphorylates type I receptor (TβRI, such as activin receptor-like kinase, ALK), and subsequently activates the Smad signaling cascade. Smad2 and Smad3 proteins are activated by TβRI-induced phoshorylation, leading to further Smad2/3 interaction with Smad4. Such complex is subsequently translocated to the nucleus where it acts as a transcription factor [100]. Depending on the cellular context, TGFβ is associated with both pro- and anti-angiogenic properties [96]. Pro-angiogenic effects of TGFβ are a combination of direct and indirect effects as TGFβ can induce VEGF production, leading to the induction of sprouting, and as TGFβ can also attract macrophages, which in turn secrete many pro-inflammatory and pro-angiogenic factors [100]. *In vitro* applied TGFβ induces apoptosis in ECs, opposing VEGF's pro-survival effects. However, TGFβ-induced apoptosis is necessary for vessel formation, thus abrogation of this signaling causes an abnormal vasculature [101]. In some malignancies, such as pancreatic cancer, TGFβ induces the expression of thrombospondin-1, a potent angiostatic factor. Perturbation of TGFβ signaling in this case leads to an enhanced angiogenesis [34,102].

1.4.2.6. Platelet-derived growth factor signaling

Platelet-derived growth factor (PDGF) is an important player in the vessel wall maturation. PDGF can exist as homo- or heterodimers composed of isoforms A, B, C and D. These growth factors are secreted by ECs in order to recruit pericytes and smooth-muscle cells to the vessel, and to subsequently stabilize the vessel structure. Upon binding of PDGFs to their PDGF receptors (PDGFRα and β), belonging to the RTKs, these receptors dimerize and autophosphorylate. Autophosphorylation reveals docking sites for the SH2-domain-containing signaling molecules, including tyrosine kinases of the Src family, PLC-γ and the GTPase activating protein (GAP) for Ras. Activation of these signaling cascades leads to an enhanced migration, proliferation and cell survival [93,103]. In CRC, these PDGF receptors are mainly present in CAFs and pericytes and their expression is correlated with a poor prognosis [103,104].

1.4.2.7. Angiogenin

Angiogenin (ANG) is a potent stimulator of angiogenesis that interacts with ECs and smooth-muscle cells to exert proliferative, pro-invasive and pro-migratory effects. ANG stimulates degradation of basement membrane via binding to αSMA at the EC or smooth muscle cell surface.

This interaction leads subsequently to activation of proteolytic cascades with generation of plasmin and other proteases that degrade laminin, fibronectin and other components of basement membrane. Although ANG belongs to the ribonuclease superfamily, it has relatively weak ribonucleolytic activity, yet necessary to induce the pro-angiogenic actions. ANG was shown to undergone nuclear translocations, where it enhanced ribosomal RNA transcription via binding to CT-rich angiogenin-binding elements. Besides, ANG was shown to activate several signal transduction cascades via interactions at EC's cellular membrane. These actions were shown to stimulate the extracellular signal-related kinase (ERK1/2) MAPK and Akt, resulting in enhanced EC proliferation. Elevated levels of ANG were detected in various types of cancers such as breast, prostate, liver, and colorectal cancer, suggesting its close association with tumor development [105,106].

1.4.2.8. ECM components and ECM remodeling in angiogenesis

The ECM and its remodeling are key players during all phases of angiogenesis. Although structural support is key function of ECM, during angiogenesis the ECM is also an important signal mediator. In the initial phase of angiogenesis, an activated tip cell adheres to the ECM via cell-surface integrins, leading to the activation of ERK1/2 MAPK signaling cascade, which is crucial for EC proliferation, survival and migration. During sprouting ECs must jointly undergo morphogenesis, which is provoked by, among other molecules, collagen type I and integrins. This leads to the activation of Rho, Src and p38 MAPK pathways and results in an enhanced EC contractility and acquisition of a spindle-shaped morphology. Moreover, together with the ECM proteases, collagen and fibrin are important players in lumen formation. ECM proteolysis driven by membrane-anchored pericellular collagenase MT1-MMP is critical in facilitating expansion of EC tubes and formation of vascular guidance tunnels. The family of MMPs consists of over 20 zinc-dependent metalloproteases specialized in degradation of various ECM proteins. Because of their proteolytic activity, MMPs mediate the release of many angiogenic factors (including VEGF and bFGF) in the ECM. MT1-MMP together with MMP-2 and its important negative regulator, tissue inhibitor of proteinase (TIMP)-2, are required for ECM remodeling at the leading front of an expanding sprout [107-109]. Different proteases have been shown to have opposite roles in the angiogenesis: a desintegrin and metalloproteinase (ADAM) and a desintegrin and metalloproteinase with thrombospondin motifs (ADAMT). ADAM-17 enhances angiogenesis via the activation of MMP-2, while ADAMTs can have pro-apoptotic effects on ECs [109]. Another important ECM enzyme system, the serine protease urokinase-type plasminogen activator (uPA) cascade, has a recognized role in wound healing, angiogenesis and cancer invasion, not only via ECM remodeling but also via intracellular signaling. uPA binds to its receptor (uPAR) leading to the cleavage of plasminogen and the subsequent release of active serine protease plasmin, which in

turn activates other proteinases, including MMPs and uPA itself, and as such drives degradation of many ECM structural proteins. Moreover, uPA-bound uPAR influences cell adhesion and migration via interaction with several proteins, including integrins and vitronectin. Furthermore, an uPAR interaction with integrins leads to the EGFR-dependent cell proliferation. Lastly, plasminogen activator inhibitors (PAI)-1 and -2, play important roles in the negative regulation of the uPA system, and as such are associated with anti-angiogenic functions [110].

1.5. Cancer invasion and metastasis

Cancer invasion and subsequent metastasis belong to the hallmarks of cancer leading to cancer dissemination. Metastasis forms the main cause of cancer mortality [8]. The invasion-metastasis cascade is a multistep process that begins when the cancer cells lose their cell-to-cell connections, and display increased proteolytic activity via production and release of proteolytic enzymes. Further, with the help of adjacent cells from the tumor microenvironment, cancer cells can migrate and spread to neighboring tissues and blood vessels, to eventually extravasate and colonize a distant target niche [13].

1.5.1. Mechanisms of cell invasion

Cell invasion is a key process in cancer progression. Depending on the tissue and the cell type, two major types of invasion are proposed: collective and individual. The latter invasion type proceeds via mesenchymal or amoeboid cells [13,111]. Moreover, during invasion, cancer cells can undergo morphological and phenotypical conversions, such as EMT, mesenchymal to amoeboid transition (MAT) or collective to amoeboid transition (CAT) [13,112,113].

The collective cell invasion is characteristic for epithelial tumors, such as breast, CRC and endometrial cancer. In this type of invasion, cell-to-cell contacts remain unaffected and the cytoskeletal activity, in combination with multicellular coordination of polarity generates traction forces, resulting in collective cell migration (Figure 8A). Moreover, collective cell invasion requires ECM remodeling and rearrangement of the basement membrane [114,115].

The mesenchymal single cell invasion was reported in fibrosarcoma, melanoma and glioblastoma. In epithelial cancers, mesenchymal single cell invasion occurs via EMT and subsequent detachment of single cells from a cell cluster (Figure 8B) [13,111]. Mesenchymal cell invasion comprises several steps leading to a change in cell shape and rearrangement of the tissue through which the cell migrates. First, the actin cytoskeleton becomes polarized, forming a protruding front and a retracting rear end, which result in a polarized cell shape and a formation of leading extension. Next, the leading extension interacts with the extracellular components, resulting in a focal cell adhesion and the generation of traction forces. Further, cell-surface proteases contribute to ECM degradation, which facilitates cell migration and uncovers embedded growth factors and other molecules accelerating the process. Further steps involve the activation of myosin II and the generation of actomyosin-based contractile tensions within the cell. The final step is a continuation of contraction and iteration of the cell adhesion events [111].

The invasion of single amoeboid cells is the fastest migratory phenotype and was reported in lymphoma, melanoma, and several epithelial cancers. The amoeboid cell invasion is a result of cell polarity loss, loose connections with the ECM and the ability of chemotaxis [111,116]. The

amoeboid cells have a round morphotype, lack stress fibers formation and weakly affect the ECM. Instead, amoeboid cells use actomyosin mechanical forces to squeeze between narrow spaces and gaps within the ECM and do not require proteolytic activity (Figure 8C) [13,111].

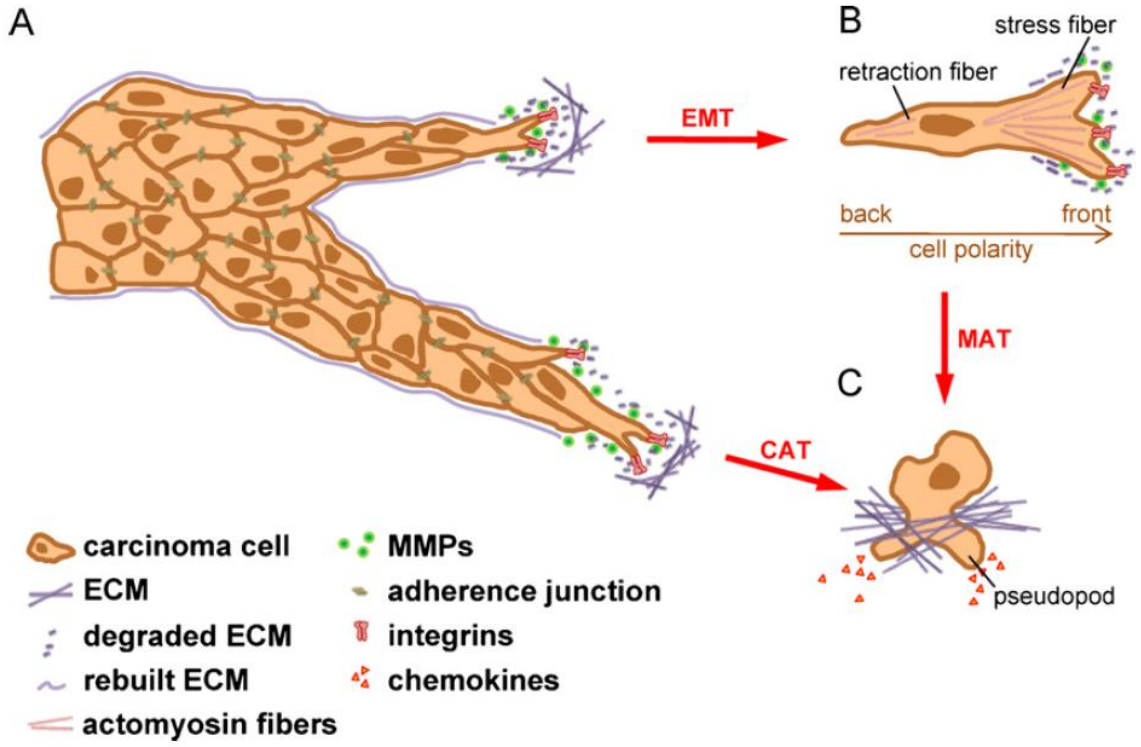

Figure 8. Mechanisms of cancer cell invasion. (A) During the collective cell migration, tip cells at the invasive margin of the tumor promote ECM remodeling and cell-matrix adhesions. The following cells within a sheet or a strand maintain most of their epithelial characteristics including the cell-cell connections. (B) Single cancer cells are able to detach from the cluster by undergoing an EMT (which can occur due to TGFβ-driven downregulation of E-cadherin and cytoskeleton rearrangements). (C) In amoeboid cell invasion, which can occur via MAT or CAT, the cancer cell is devoid of polarity and relies on protease-independent chemotaxis. Source [13].

It should be emphasized that above described modes of cell migration present the extreme forms and in reality the occurring migration processes range between these two opposed forms. Various combination of both intrinsic and environmental factors have an impact on the final outcome of the invasion mode, and moreover, cancer cells demonstrate a substantial plasticity in terms of adapting their migration mode [111]. The intrinsic factors determining the migration mode include the volume and plasticity of the cytoplasm and the nucleus, organization of the cytoskeleton, and activity of the small GTP-ases RhoA, Rac, and Cdc42 being key regulators of cell motility [117]. RhoA-controlled increased contractility is associated with amoeboid-like migration, while lower contractility together with increased cell adhesion favor mesenchymal-like migration mode. Furthermore, as evidenced by intravital studies, the microenvironmental factors, such as stromal collagen network organization, determine the migration mode and its dynamics. Cancer cells, amoeboid-shaped, as well as mesenchymal morphotype use collagen fibers bundles as

routes for directional migration. Moreover, proximity of blood vessels and macrophages is associated with increased cancer cell motility [118,119]. However, the confinement level of the surrounding microenvironment (including pores in the ECM or tunnel-like tracks) seems to determine the acquisition of amoeboid versus mesenchymal migration mode. In the confined spaces with pores diameter lower than nuclear diameter, cells acquire mesenchymal-mode of migration requiring proteolytical activity and adhesion. In contrast, cells using the pre-existing tracks or pores with higher diameter than the nucleus diameter acquire rather amoeboid-type migration and the exact mode depends on cell contractile abilities and levels of adhesion [120].

1.5.2. Epithelial to mesenchymal transition

Epithelial to mesenchymal transition (EMT) occurs naturally during embryo development and in response to injury, but also in pathological states, such as fibrosis. Moreover, EMT is an important process that drives invasion of many epithelial cancers, including CRC [13,112].

During EMT epithelial cells acquire typical characteristics of mesenchymal cells, losing meanwhile many of their epithelial properties. The normal epithelium is composed of polarized layers of cells (defined as inside - basal and outside - apical), that provide tight cell-to-cell connections (by tight, gap and adherens junctions). At the basal surface, cells maintain their matrix-binding to the base membrane and its component, laminin. The actin cytoskeleton of epithelial cells is apico-basally polarized with a peripheral organization. In contrast, mesenchymal cells, are motile, of an elongated shape and with a front-to-back polarity, creating a scattered network instead of a layer. The mesenchymal cells are characterized by a dense actin filament network and the presence of vimentin as an intermediate filament. Moreover, the matrix-binding sites are densely distributed on their cell surface [16,112]. The loss of expression or function of the cell-to-cell adhesion glycoprotein epithelial cadherin (E-cad) was described as a key step during EMT. Several known transcription factors such as zinc finger E-box-binding homeobox (ZEB), Slug and Snail are known to repress expression of E-cad via an inhibitory activity on its gene promoter. Other factors, such as Twist and FoxC2 are proven to repress E-cad transcription indirectly. Besides their role in repressing E-cad expression, these EMT-inducing factors contribute to the acquisition of an overall mesenchymal phenotype, cell proliferation and survival. Another crucial event during EMT is the loss of epithelial polarity. In that respect, protein complexes maintaining polarized epithelium, namely Par, Crumbs and Scribble, are also negatively regulated by ZEB and Snail factors suppressing their protein expression. This loss of polarity is also indirectly affected by TGFβ which stimulates ZEB and Snail expression. Furthermore, ZEB and Snail induce the expression of MMPs and, as such contribute to the basement membrane degradation and subsequent cell invasion.

Besides TGFβ, also hypoxia and several signaling cascades, including FGF, EGF, HGF, Wnt/β-cat, and Notch, are known to induce EMT [112,121].

1.5.3. Metastasis

Metastatic tumors can be formed via direct primary cancer local expansion, a spread to nearby tissues or organs, or a distant spread via blood or lymphatic systems. Lungs, liver, bones and brain are the most common metastatic destinations. CRC most often metastasizes to the peritoneum, liver and lungs [5]. Cells that disseminated from the primary tumor and spread to the blood system are called circulating tumor cells (CTCs) and can be detected in patients with advanced primary carcinomas. However, thanks to mechanical forces and presence of immune cells, such as NKs in blood, only a small percentage of CTCs is able to survive and actually produce metastases. However, CTCs can counteract those dangers by expressing tissue factor proteins that attract aggregating platelets and, as such contribute to cancer cell protection and survival. The metastatic environment offers new challenges to the CTCs. Before a micrometastasis is formed a CTC must lodge, survive, extravasate and colonize the new niche [122,123]. According to the modern context of the "seed and soil" hypothesis, proposed first in 1889 by Stephen Paget, CTCs (seeds) can colonize only certain tissues (soil) that allow cancer cells to adhere and proliferate, while other sites would be less accepting [124,125]. The successful colonization depends not only on the cancer cells' abilities to adhere and proliferate, but also relies on the specific growth factors' availability within the ECM and the recruitment of the stromal cells, such as myofibroblasts and endothelial cells, to support micrometastatic growth [122,123].

Mounting evidence suggests that cancer cells can disseminate also during the early stages of tumorigenesis, thus before the initial diagnosis. Recent research in HER2-driven mouse breast cancer model showed that such early dissemination occurred soon after cancer initiation, before the switch to intensive cancer cell proliferation, and was regulated by cell density, activity of HER2 and progesterone signaling. In this case, progesterone acting via its receptor stimulated an early cancer cell invasion via up-regulating *Rankl* and *Wnt4* gene expression [14,15].

Metastasis often develops years after surgical removal of the primary tumor. Recent studies on latency competent cancer (LCC) cells showed that disseminated cancer cells that survived the initial therapy are able to survive in distant organs in a latent state. LCC cells remain in a stem cell-like state, undetected by host's NK cells, due to very low division rate controlled by Wnt pathway inhibitor, DKK1. Such quiescent cells can remain in the host's organs for years [126].

1.6. Cellular cross-talk in the cancer microenvironment

The exchange of signals between particular cellular components is essential for the tumor progression. The understanding of these interactions between the cancer cells and the stromal cells creates opportunities for new diagnostic and therapeutic approaches. Moreover, recruited CAFs affect other cellular components, such as endothelial cells, facilitating cancer angiogenesis and its subsequent progression [68].

1.6.1. Interactions between cancer cells and myofibroblasts

Myofibroblasts recruited by cancer cells (CAFs) affect cancer progression through release of soluble factors, exosomes or via cell-to-cell signaling. The constant dialogue between cancer cells and CAFs promotes multiple hallmarks of cancer [8,127].

TGFβ and PDGF are key factors produced by cancer cells to recruit CAFs. TGFβ is a strong chemoattractant of fibroblasts, promotes transdifferentiation of fibroblasts and myofibroblasts, and is an important inducer of αSMA expression, the major component of the contractile apparatus [35,128]. PDGF is also a potent chemoattractant for mesenchymal cells and stimulates cell proliferation. Although PDGF is not a direct factor to drive fibroblasts transdifferentiation, it stimulates local macrophages to produce TGFβ [35,103].

Transmembrane glycoprotein neural-cadherin (N-cad) is a molecule with the ability to establish cell-to-cell connections, but is also a path-finding molecule and an important driver of cancer invasion. Cells undergone EMT lack E-cad expression in favor of N-cad, which is also abundantly expressed on the surface of mesenchymal cells, including CAFs. Moreover, soluble N-cad (sN-cad, a cleaved extracellular domain of the molecule) can be released by cells and embedded within the ECM [35]. (s)N-cad is able to interact with the FGF receptor leading to prolonged ERK MAPK activation and subsequent cell migration [129,130]. TGFβ released by cancer cells is a strong stimulator of N-cad expression in CAF filopodia. The cytoplasmic domain of N-cad connects with the actin cytoskeleton via catenins and upon cadherin-established extracellular contacts it translates the signal intracellularly into actin-mediated changes in cell shape and cell adhesion, resulting in cell motility [35,131,132].

CAFs produce and release a multitude of molecules, which facilitate cancer progression, including ECM structural proteins (collagens, proteoglycans, tenascins), proteases (MMPs, ADAMs, uPA), growth factors (EGF, HGF, FGF, IGF-1, SDF-1, VEGF), and cytokines (IL6, RANTES/CCL5). These proteins play a direct or indirect but essential role in promoting cancer cell EMT, proliferation and invasion [76]. CAFs-released MMP-2 and MMP-9 are specialized in degrading collagen type IV and laminin, which are the main structural components of the base membrane [133]. Hepatocyte growth factor/scatter factor (HGF/SF) is a mitogenic and motogenic protein that targets both

endothelial and epithelial cells and is an important mediator of the EMT during organ development and regeneration. HGF is released by stromal cells in a latent form as a pro-HGF that can be activated by ECM proteases, such as uPA. HGF binds to its receptor HGFR, also known as c-Met, a proto-oncogenic product of the RTK family. Upon binding, c-Met undergoes autophosphorylation leading to the activation of several molecular cascades, such as PI3K and Wnt, and subsequently resulting in cell proliferation, migration, survival, morphogenesis and induction of angiogenesis [134]. Secreted by CAFs, HGF and TNC together, act as important stimulators of invasion for colon cancer cells via RhoA and Rac signaling [77]. C-C chemokine ligand 5 (CCL5), also known as regulated on activation, normal T-cell expressed and secreted (RANTES), is a molecule secreted by cancer cells or by CAFs and plays an active role in attracting immunosuppressive cells, such as Tregs and macrophages. Its receptor CCR5, which is a G-protein coupled transmembrane protein, mediates CCL5 signaling, leading to the activation of integrins, actin cytoskeleton polarization, F-actin formation and the subsequent cell migration through PI3K/Akt and NFκB signaling [135,136]. Both CCL5 and CCR5 are overexpressed in CRC at primary sites, as well as at the metastatic sites [137].

1.6.2. Interactions between CAFs and endothelial cells

Factors released by CAFs affect not only the cancer cells, but can also attract ECs and facilitate angiogenesis [138]. Previous studies have shown that in a xenograft mouse model breast cancer cells mixed with CAFs created larger tumors with more complex vasculature, as compared to tumors with primary human fibroblasts added instead of CAFs. Secreted by CAFs stromal cell-derived factor (SDF)-1 was proven to be a potent chemoattractant for endothelial progenitor cells in order to create blood vessels *de novo*, in a process of vasculogenesis [139]. CAFs are known to produce potent pro-angiogenic factors including VEGF, bFGF, PDGFs, TGFβ, HGF, connective tissue growth factor and IL-8, which create favorable conditions for EC migration, proliferation and further vessel expansion. Furthermore, the MMPs released by CAFs and the subsequent ECM remodeling facilitate not only the migration of cancer cells, but also enhance expansion of ECs. In addition, cytokines and chemokines released by CAFs, modulate inflammatory responses within the local microenvironment, leading to the recruitment of macrophages, which produce many pro-angiogenic factors independently [140]. Colon cancer-derived CAFs secrete IL-6, and this release is stimulated even more by colon cancer cells. Moreover, IL-6 was shown to enhance VEGF expression and secretion by colon fibroblasts [141]. Although, ECs' influence on their surrounding is not clearly defined besides their structural role, the ECs can affect the local environment via release of proteolytic enzymes and subsequent ECM remodeling as well as via transdifferentiation into myofibroblasts [140].

1.7. Cancer stroma and the therapy resistance

Resistance to tumor therapy can be divided into two major types: *de novo* and acquired resistance. Whereas an acquired drug resistant phenotype develops as a result of an accumulation of genetic changes over time, *de novo* resistance is mediated by the cancer environment itself and is a quick transient response induced by signaling cross-talk between cancer cells and stromal cells [142].

Acquired therapy resistance of cancer cells can occur via different mechanisms and can exist for each type of cytotoxic therapy or targeted pathway. Multidrug resistance (MDR) occurs when a resistance to one drug can lead to or coincide with a resistance to another drug, often due to overlapping mechanisms. Cancer cells can counteract traditional cytotoxic agents via decreased intake of the drug into the cell, by an increased efflux or may be conferred by more complex genetic and/or epigenetic changes that affect drug sensitivity [143]. Mechanisms involved in CRC resistance to traditional cytotoxic drugs include acquired resistance to 5-FU via an increased expression of the 5-FU target protein, thymidylate synthase [144]. Capecitabine resistance can occur due to a decrease of thymidine phosphorylase expression, resulting in an impaired formation of the drug's active metabolite [145]. An increase in the MDR protein expression leads to an increased efflux of irinotecan and oxaliplatin [146,147].

Various mutations, upregulations or activations of the downstream effectors within specific signaling pathways can cause resistance to targeted therapy [143]. Resistance to EGFR antagonists can occur via activating mutations of KRAS, PI3K and BRAF, but also via PTEN loss of function mutation or via amplification of genes encoding for human epidermal growth factor receptor (HER)2 or c-Met [148-152]. An increased activity of HIF-1, an increased expression of PlGF, IL-8, and VEGF-D are some of the mechanisms counteracting VEGF antagonists [153-156].

Various kinds of cancer therapy, including surgery, chemo- and radiotherapy affect not only cancer cells but inevitably also the surrounding stroma. Wounds created during surgery trigger a healing process, creating a favorable niche for cancer cells that were not removed during the surgical intervention [76,157]. Similarly, obtaining biopsies for histopathological examination creates wounds within the tumor mass. In the areas along the biopsy track in breast cancer, CAFs were shown to produce higher amounts of uPA compared to the intact parts of the tumor [158].

Since decades, radiotherapy has played an important role in cancer treatment. Ionizing radiation (IR) has been shown to affect non-cancerous tissues by triggering fibrosis and transition of fibroblasts into myofibroblasts. Also the cancer stroma is susceptible to IR. In fact, changes made to the stromal components contribute to further therapy resistance and cancer recurrence, and the IR-triggered inflammation drives CAFs to produce growth factors, cytokines and proteases to a

higher extent. Moreover, IR causes destruction of blood vessels, especially the microvasculature. IR affects EC functionality, by triggering EC apoptosis and detachment from the basement membrane, which increases vessel permeability. These changes contribute to the inflammatory and fibrotic responses within the tumor. Moreover, the vascular damage leads to tumor hypoxia, which in turn plays a key role in radiotherapy resistance. Oxygen shortage within the tumor reduces the production of reactive oxygen species that under normoxia trigger DNA damage leading to apoptosis. Moreover, hypoxia-induced HIF-1 activity, was also shown to promote radioresistance [76,159,160].

Furthermore, a growing body of evidence suggests that radiotherapy and chemotherapy affect healthy tissues to form a pro-metastatic environment. Toxic tissue damage, as a side effect of therapy leads to the upregulation of multiple chemokines, growth factors and other molecules, such as SDF-1 and sphingosine-1 phosphate. In a normal situation these factors attract stem cells to the injured sites as part of the healing process, however, they may also be exploited by cancer cells which survived the treatment, and as such, may facilitate the formation of micrometastases [161].

Environment-mediated drug resistance (EMDR) is *de novo* drug resistance, associated with interactions between cancer cells and the stromal microenvironment, resulting in a protection of cancer cells from the effects of different cytotoxic therapies [142]. Many studies concerning the role of stromal cells in cancer chemoresistance have recently been reported. In a model of colorectal CSCs, chemoresistance was increased by CAFs. The chemotherapy-induced CAFs promoted *in vivo* tumor growth and self-renewal of colorectal CSCs via the production of multiple cytokines and chemokines (including IL-17) in CAFs in a response to chemotherapy. Further studies with exogenous IL-17 displayed its growth- and invasion-stimulating effects on CSCs [162]. In the studies performed on BRAF-mutant melanoma in a co-culture system, stromal cells protected cancer cells from the PLX4720 RAF inhibitor via HGF secretion. Stromal-derived HGF activated the c-Met receptor and subsequently led to activation of MAPK and PI3K/Akt signaling cascades [163]. Furthermore, recently published results showed that the soluble factors produced by colon cancer-derived CAFs in the presence of chemotherapy, induced a nuclear translocation of Akt, p38 MAPK, and survivin in cancer cells, resulting in a promotion of colorectal cancer cell survival and proliferation [164]. In co-cultures of a head and neck squamous cell carcinoma (HNSCC) cell line and CAFs, a growth-inhibitory effect of EGFR antagonist cetuximab was neutralized, and a similar effect was observed in an experiment using CAF-derived conditioned medium. The cross talk between HNSCC and CAFs led to an upregulation of MMP-1, and since this drug resistance was abolished in the presence of an MMP-1 inhibitor, an important role of MMP-1 in the mechanism of chemoresistance was suggested [165]. CAF-mediated resistance to hormonal

therapy was recently also reported, as *in vivo* and *in vitro* evidence has shown that CAFs derived from luminal breast cancer induced tamoxifen resistance in cancer cells via the stimulated release of IL-6 [166].

Owing to its undeniable role in cancer progression and protection, cancer stroma also became a target in the cancer therapy and diagnostics [167]. RNA signatures of tumors (the transcriptome) became an important tool in the cancer classification and in establishment of the prognosis. The RNA information derives not only from cancer cells but also from the surrounding stroma, and in fact, most genes indicating a poor prognosis are of the CAF origin [168]. Furthermore, CAFs and their cross-talk with the cancer cells, other stromal cells, and with the ECM within the tumor microenvironment, became the target in therapy [167]. Nintedanib (BIBF 1120) is a tyrosine kinase inhibitor designed to target the tumor microenvironment. Thanks to its broad spectrum, nintedanib blocks the activity of VEGFR, PDGFR and FGFR by competitive binding to the ATP pocket of the respective receptors [169]. Targeting TGFβ signaling strongly influences the interaction networks within the cancer microenvironment. Galunisertib (LY2157299), an inhibitor of the TGFβ receptor is currently undergoing phase II clinical trials for multiple cancers [170]. Furthermore, CAFs have been studied as a tool in cancer treatment. In the ecological trap model, CAFs or their secreted proteins could be used as bait for cancer cells. In a mouse model, CAFs encapsulated in the alginate-gelatin microparticles promoted adhesion of disseminated cancer cells to the microparticles' surface [171]. Current anti-cancer research focuses on developing similar methods via sabotaging stromal myofibroblasts cell function.

2. Glucocorticoid receptor: nature and the mechanism of action

The glucocorticoid receptor (GR) is a steroid hormone (SH) receptor. Together with its closest relatives mineralocorticoid (MR), progesterone (PR), androgen (AR), and estrogen (ER) receptors, GR belongs to the nuclear receptor (NR) superfamily of proteins (Figure 9). These receptors, upon binding their ligands in the cytoplasm, are translocated to the nucleus, where they bind DNA and act as transcription factors to control many major functions of the organism [172]. GR is expressed in almost every human tissue, and its actions control processes involved in development, glucose metabolism and immune responses [173,174].

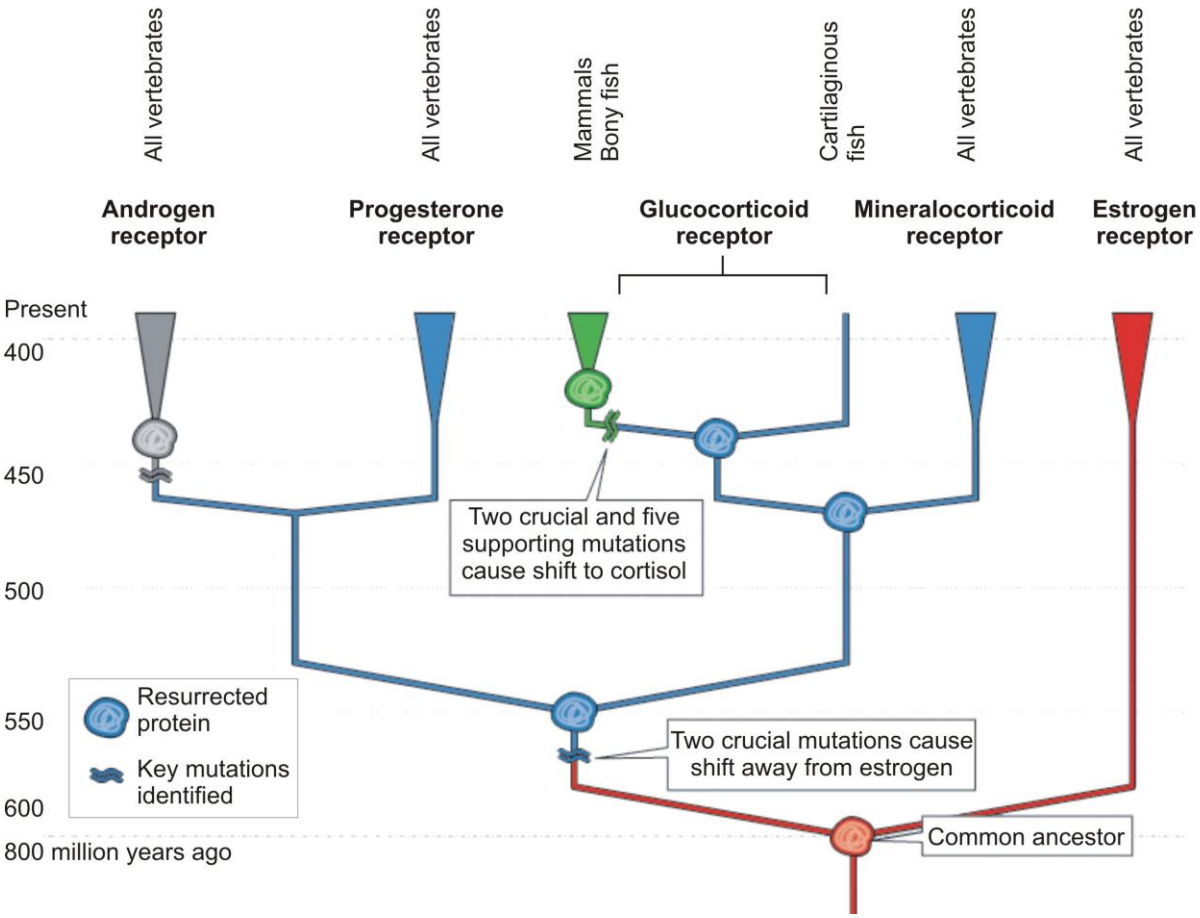

Figure 9. Evolution of the steroid hormone receptors. The vertebrate SH receptors evolved from a common ancestor. Works conducted by the group of prof. J. Thornton resulted in the resurrection of ancient SH receptor proteins and revealed the key events driving their evolution. A common ancestor of the GR and MR could bind to both the cortisol and aldosterone. Over the course of approximately 20 million years GR became solely sensitive to cortisol in the bony vertebrates. Adapted from [175] and [176].

2.1. Organization of the glucocorticoid receptor

The GR protein is composed of three major domains: the N-terminal transactivation domain (NTD), the central DNA-binding domain (DBD) and the C-terminal ligand-binding domain (LBD) (Figure 10C). The DBD and LBD regions are linked by a flexible hinge region [177]. The NTD region holds a strong transcription activation function (AF1) by interacting with the basal transcription machinery (BTM) and co-factors. Moreover, the NTD houses multiple sites for the post-translational modifications (PTMs). The DBD has two zinc finger motifs, able to bind to the target DNA regions containing the glucocorticoid-responsive elements (GREs) and is the most conserved domain across the NR family. The LBD contains a hydrophobic pocket which binds the ligands, glucocorticoids (GCs), and it also has an AF2 which binds certain ligand-dependent co-factors. Furthermore, GR contains two nuclear localization motifs: an NL1, which is at the DBD/hinge region junction and an NL2, located within the LBD [178].

2.1.1. Alternative splicing of the GR gene

The human GR gene (NR3C1) is located at chromosome 5 and comprises 9 exons (Figure 10A). The NTD is encoded by the exon 2, DBD by exons 3 and 4, and the hinge region and the LBD are encoded by the following exons. The alternative splicing occurring in the exon 9, near the end of the primary transcript, results in GRα and GRβ isoforms [178,179]. Moreover, the alternative splicing in the intron between exons 3 and 4 yields another GR isoform, GRγ, containing a single arginine insertion within the DBD. GRα is the main isoform that binds GCs and functions as a transcription factor, whereas GRβ, although localized in the nucleus, is not capable of activating GC-responsive reporter genes, since it does not bind GCs [180]. Nevertheless, GRβ can bind to GREs, therefore when co-expressed with GRα it acts as an inhibitor of GRα actions. Moreover, GRβ's heterodimerization with GRα and competition for co-factors potentially contributes to an inhibitory function associated with GC resistance [178]. GRγ binds GCs and displays similar properties as GRα, but its functionality is strongly impaired and its transcriptional profile differs from GRα. GRγ's expression, similarly to GRβ, is associated with a GC resistance [181,182]. Furthermore, two other splice variants of GR have been reported: GR-A and GR-P. GR-A has a truncated LBD and its function is currently unknown. GR-P, however, has been described to interfere with GRα transcriptional activity, depending on the cellular context [178].

2.1.2. Translational isoforms of GR

Due to a different initiation of translation of its mRNA, GRα exists in multiple translational isoforms (Figure 10B). From the eight AUG start codons present in exon 2 of the GR gene, eight different variants (GRα-A, GRα-B, GRα-C1, GRα-C2, GRα-C3, GRα-D1, GRα-D2, GRα-D3) can be formed with progressively shorter NTDs. GRα-A, translated from the first AUG codon is the

protein of full-length 777 amino acids, and together with other isoforms it is able to bind GCs and target the GREs. However, other differences between these various isoforms are reported. GRα-A, -B, and -C reside in the cytoplasm, whereas GRα-D has a nuclear localization. Interestingly, less than 10% of genes are targeted by all GRα isoforms, indicating a specificity of action of each receptor subtype [183,184]. In fact, GRα-C showed the most pronounced activity in the transcription of reporter and endogenous genes, while GRα-D was the least active. GRα-D has the most truncated NTD, presumably explaining its considerably impaired transcriptional activity. Importantly, expression of various GRα isoforms differs depending on the tissue and cell type. GRα-A and -B are most abundantly present, however, immature dendritic cells are rich in GRα-D and trabecular meshwork cells in the human eye are characterized by expression of GRα-C and GRα-D [173,185,186].

2.1.3. Post-translational modifications of GR

Post-translational modifications (PTMs) are covalent modifications of proteins, resulting from an enzymatic activity. PTMs enrich proteins by adding new functional groups such as phosphate, acetyl or methyl groups. GR's PTMs strongly contribute to the receptor's function and activity (Figure 10D) [178]. The most frequent PTM of proteins is phosphorylation [187]. In GR, phosphorylation can occur at several serine residues located in the NTD (Ser-113, -134, -141, -203, -211, -226, -404). MAPK, cyclin dependent kinases (CDKs) and glycogen synthase kinase (GSK)-3β belong to the major group of kinases that phosphorylate GR and contribute to changes in the GR's transactivation activity [178,188]. Phosphorylation at Ser-211 enhances GR's capability to activate transcription, whereas at Ser-226 impairs this function [189]. Moreover, Ser-404 phosphorylation is crucial in diminishing GR's ability to activate or suppress certain target genes [190]. Furthermore, phosphorylation at Ser-211 residue, located in the AF1 domain, is important to facilitate the co-factor binding [191].

Other PTMs of GR include ubiquitination, sumoylation, and acetylation. Ubiquitination of the GR occurs at a conserved lysine (Lys-419) residue located at the end of the NTD region, leading to protein degradation by the proteasome complex. Mutation at the Lys-419 impairs ligand-dependent GR downregulation, resulting in a receptor accumulation and an enhanced target gene transactivation [192]. SUMOs (small ubiquitine-related modifiers) are attached to other target lysine residues (Lys-277, -293, -703). Sumoylation enhances GR's binding to a ligand, but also plays a role in the recruitment of certain co-factors, and as such, modulates GR's transcriptional activity [193]. GR acetylation at residues Lys-494 and -495, located in the hinge region is associated with an impaired GR interaction with NFκB, and a subsequent inhibition of GR's anti-inflammatory properties [194].

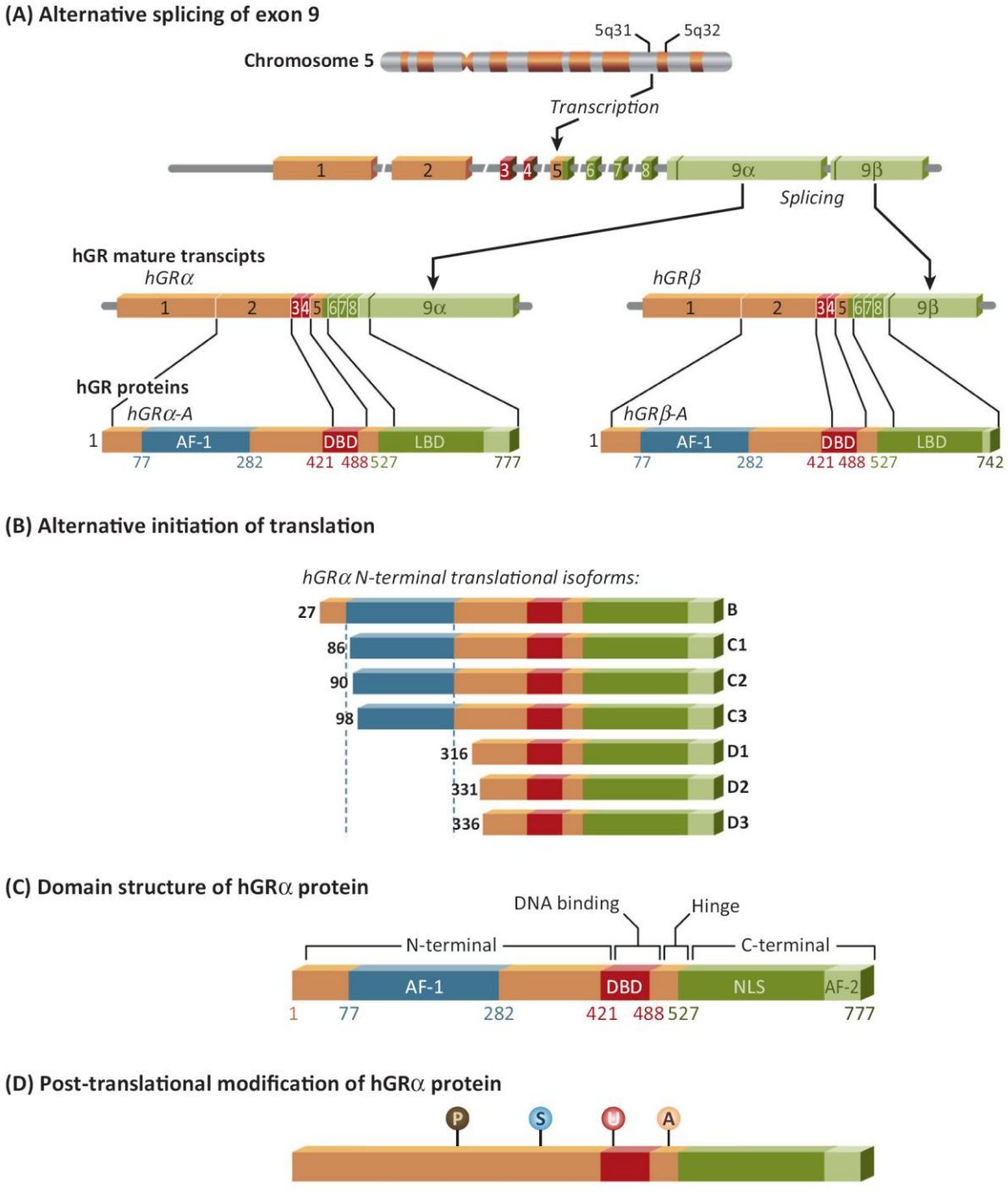

Figure 10. Structure of the human glucocorticoid receptor and its variants. Description in the text (section 2.1). Figure adapted from [195].

2.1.4. Membrane-bound glucocorticoid receptor

It is believed that most of the GR-mediated, mainly the anti-inflammatory and immunosuppressive, actions result from the activation of cytosolic GR (cGR). There is, however, a solid body of evidence that a membrane-bound GR (mGR), plays a significant role in a non-genomic signaling, which constitutes a very rapid response to a stimulus [196]. For the first time identified in 1987 and further investigated in the human lymphoma and leukemia cells, mGR is most probably encoded by the same gene as the cGR [197,198]. The expression of mGR was shown to correlate with a GC-induced apoptosis triggered in the lymphoid cells [199]. Moreover, GR presence in rat postsynaptic membranes in the lateral amygdala suggested mGR's influence on the synaptic transmission plasticity, which is related with fear and emotional memory [200]. Recent studies showed mGR expression in human monocytes and B lymphocytes, and a treatment with a pro-inflammatory molecule lipopolysaccharide (LPS) correlated with the number of mGR-positive monocytes. Moreover, mGR concentration was increased in the monocytes of patients with autoimmune afflictions, such as rheumatoid arthritis, lupus erythematosus and ankylosing spondylitis [201-203]. Ligand-activated mGR leads to an intracellular signal transduction via MAP kinases, however, the detailed mechanism of mGR actions is to date not fully understood [196].

2.2. Glucocorticoids

The name glucocorticoid (GC) combines the terms “glucose”, “cortex” and “steroid” owing to GC’s role in the glucose metabolism, origin of GC synthesis and its chemical structure. GCs belong to steroid hormones, and together with the mineralocorticoids form a group of corticosteroids – hormones produced by vertebrates in the adrenal cortex. Cortisol is an endogenous GC that binds GR in humans [204].

2.2.1. Endogenous glucocorticoids and the hypothalamic-pituitary-adrenal axis

Natural GCs are produced from cholesterol in the zona fasciculata of the adrenal glands. This process is strictly controlled by the hypothalamic-pituitary-adrenal (HPA) axis, regulated by the circadian and ultradian rhythm and also by environmental and physiological stressors (Figure 11). The hypothalamus releases the corticotropin-releasing hormone (CRH), which in turn stimulates secretion of the adrenocorticotropic hormone (ACTH) by the anterior pituitary gland. The ACTH causes induction of cortisol synthesis in the cortex of the adrenal gland and its subsequent release into the bloodstream, where more than 90% of the cortisol is bound by the corticosteroid-bound globulins (CBGs). Homeostasis in the GC release is regulated by the negative-feedback loop inhibiting the synthesis of CRH and ACTH. The unbound cortisol is a biologically active GC, which within the tissues can be converted to an inactive cortisone by the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), in a reaction reversed by 11β-HSD1 [205].

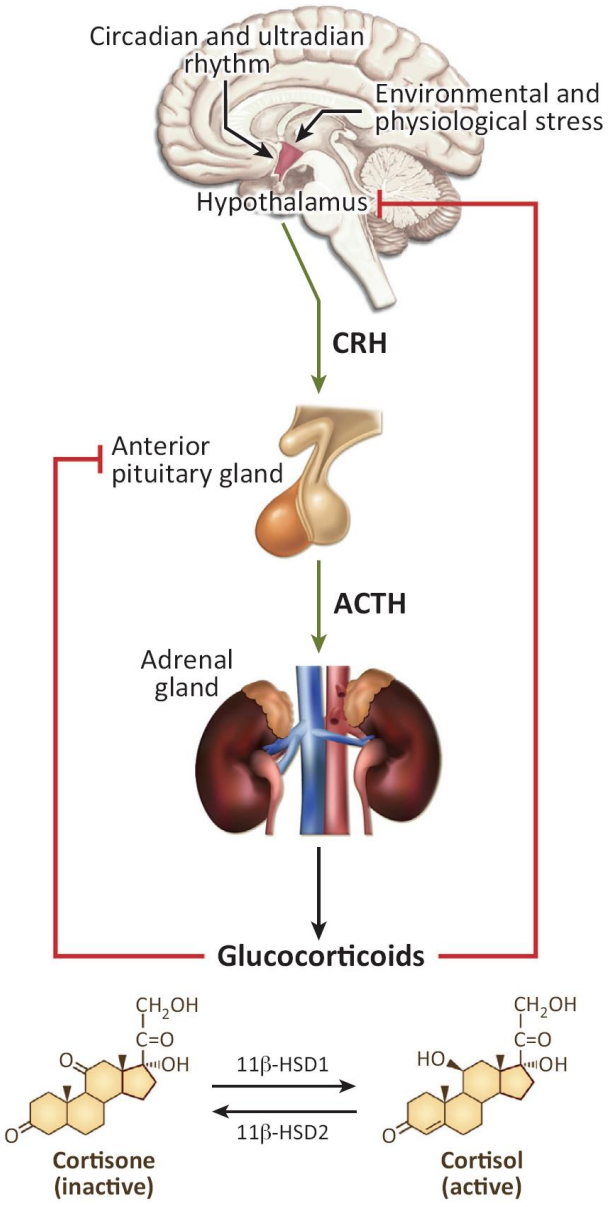

Figure 11. Schematic of the HPA-regulated levels of endogenous glucocorticoids. Description in section 2.2.1. Figure adapted from: [195].

The cortisol release in humans fluctuates during the day, reaching its peak levels in the morning and lowest levels at night. Cortisol is essential for development and whole-body homeostasis, it regulates the metabolism of carbohydrates, lipids and proteins in all major systems, including central nervous, cardiovascular, musculoskeletal, and immune system. Moreover, cortisol release is elevated following environmental or psychological stress, or during fasting, when low glucose levels are detected in the blood. Cortisol is a counterbalancing hormone of insulin, inducing gluconeogenesis and regulating ion levels, hereby contributing to the cellular pH balance. Cortisol's anti-inflammatory and immunosuppressive properties, manifested by inhibiting T-cell proliferation and by blocking the expression of multiple pro-inflammatory molecules, are highly recognized and will be discussed further [195,205].

2.2.2. Synthetic glucocorticoids

In 1949 Philip Hench with Edward Kendall and colleagues published their observation, in which the administration of adrenal cortex-derived steroid extract to patients with rheumatoid arthritis successfully stopped the progression of the disease [206]. This led to a subsequent discovery of cortisol and in 1950 the researchers, together with the chemist Tadeusz Reichstein, obtained the Nobel Prize for Physiology and Medicine. Following these discoveries, the era of synthetic glucocorticoids began, with a development of dexamethasone, prednisone/prednisolone, budesonide and other GCs exhibiting a higher potency than cortisol (Table 2) [204]. Dexamethasone (Dex), unlike cortisol, does not undergo conversion by 11β-HSD2, and moreover, the synthetic GCs are not bound by CBGs in the bloodstream, improving their bioavailability [195,207].

Table 2. Corticosteroid comparison chart. Adapted from: [208] .

Corticosteroid	Equivalent glucocorticoid dose [mg]	Potency relative to hydrocortisone	
		Anti-inflammatory	Mineralocorticoid
Hydrocortisone (cortisol)	20	1	1
Cortisone acetate	25	0.8	0.8
Prednisone/Prednisolone	5	4	0.8
Triamcinolone	4	5	<0.5
Methylprednisolone	4	5	0.5
Dexamethasone	0.75	30	<0.5
Betamethasone	0.6	30	<0.5
Fludrocortisone	-	15	150
Aldosterone	-	0	400+

2.2.3. GC-associated side effects

GCs, although potent anti-inflammatory agents considered the most efficient therapy for many inflammatory diseases, including asthma, are overshadowed by concomitant detrimental side effects. Long-term and/or high-dose administration leads to a spectrum of systemic and local adverse effects. Due to a negative-feedback loop, GCs inhibit the release of ACTH and cortisol secretion, therefore long-term treatment with GCs should be slowly reduced in order to prevent the "steroid withdrawal syndrome", characterized by lassitude, muscle pain and fever. Other systemic side effects include muscle atrophy, osteoporosis, diabetes, hypertension, and increased risk of infection. The local atopic GC administration may result in skin thinning and a delayed wound healing. Local side effects for inhaled GCs include weakness of the voice and hoarseness. Moreover, prolonged GCs treatment at the ocular level may increase the intraocular pressure and may also cause induction of glaucoma and formation of cataract [204,209].

Table 3. Factors involved in the impaired GC response. Source [174,195].

Factor	Examples
GC bioavailability	Concentration of corticosteroid-binding globulins Activity of 11β-HSD1 and 11β-HSD2
GR	Mutations in GR gene GR gene methylation GR protein level GR affinity of the ligand GR splice variants (GRβ)
GR maturation and ligand binding	Chaperones and co-chaperones levels
Post-transcriptional modifications of GR	Phosphorylation Ubiquitination Sumoylation
Transcriptional activity of GR	Interactions with other transcription factors DNA methylation Availability of co-activators and co-repressors Homologous downregulation of the GR gene

2.2.4. Glucocorticoid resistance

Limitations of GC therapy involve not only the side effects but also the occurrence of GC resistance and an overall heterogeneity in the GC responsiveness. GC resistance has become a major obstacle in the treatment of patients with inflammatory disorders, such as asthma and chronic obstructive pulmonary disease (COPD) [195]. At the molecular level, multiple genetic and environmental factors can trigger an impaired GR activity – the key cause of GC resistance. These factors include an inhibition of GR expression, an impaired GR affinity to the ligand and a reduced ability of GR to bind DNA [210]. Homologous down-regulation of the GR, caused by GC-induced

GR binding to its own gene promoter and inhibition of transcription, was suggested to be a critical mechanism of GC resistance [211]. The primary generalized GC resistance (Chrousos syndrome), a rare familial disorder, was recently linked to a point mutation in the GR gene [212]. Moreover, other factors that strongly contribute to GR's responsiveness to GCs include: the GR gene polymorphisms, the existence of GR alternative splicing isoforms, the impaired GR nuclear translocation, the increased levels of GRβ competing with GRα for co-factors, the existence of alternative GRα isoforms due to changes in translation initiation, and the occurrence of various PTMs (Table 3) [174]. Furthermore, the hypermethylation of the GR gene promoter contributes to impaired GR expression which leads to a decreased responsiveness to GCs. A significant correlation was found between GR gene methylation and exposure to stress. Individuals who experienced early life stress presented with hypermethylation of the GR gene promoter and subsequent GC resistance, causing impaired HPA axis sensitivity to negative feedback to the hypothalamus. This impaired feedback sensitivity resulted in a longer recovery of cortisol stress response, which in turn is associated with development of psychopathologies [213,214]. Additionally, the availability of endogenous GCs is regulated by the CBGs present in the bloodstream and by the activity of the 11β-HSD1/2 enzyme system, which is also regulated independently [174,215].

2.3. Mechanism of glucocorticoid receptor modulation

A functional GR protein resides in the cytoplasm in a multiprotein complex. Upon GC binding, GR is translocated into the nucleus where it acts as a transcription factor to positively or negatively regulate expression of target genes. Alternatively, GC-bound GR may act via non-genomic mechanisms which do not require changes in gene expression [178].

2.3.1. Nuclear translocation

GR, similarly to MR and AR, is continuously transported from the cytoplasm to the nucleus and back, both in presence and absence of the ligand [216-219]. Residing in the cytoplasm, newly synthesized ligand-free GR acquires a specific protein conformation, guided by the multimeric chaperone protein complex (Figure 12).

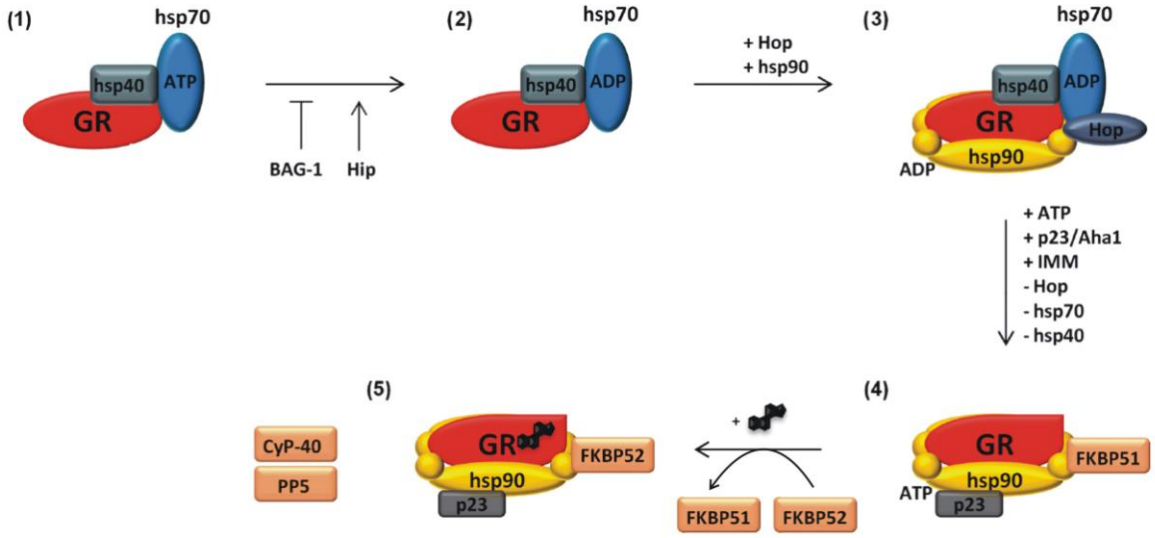

Figure 12. Schematic representation of GR maturation. 1) A newly synthesized GR binds to hsp70 in an ATP-dependent manner. 2) Binding with co-chaperones facilitates interaction with the hsp90. 3) A detachment of several proteins enables exposure of the GR's LBD and attachment of immunophilins (IMM) FKBP51 and CyP-40 and immunophilin-like protein, phosphatase (PP)5. 4) Maturation of GR led by hsp90 in an ATP-dependent manner results in the protein's high ligand affinity conformation. 5) Binding to a GC accompanies an immunophilin switch. Figure adapted from [220].

Chaperones drive GR folding from low to high ligand affinity in a process catalyzed by the ATPases heat shock protein (hsp)70 and hsp90 [220]. Hsp70 is suggested to be the first chaperone to bind to newly synthesized GR protein and its binding to GR's LBD is facilitated by a co-chaperone hsp40. A complex consisting of GR-hsp70 enables interaction with hsp90 dimers, which together with other co-chaperones (BAG-1, Hip and Hop) form a GR foldosome. During the process of GR maturation, in the presence of ATP, Hop, hsp70 and hsp40 are released from the foldosome and a co-chaperone p23 stabilizes hsp90 binding with the GR. Following the release of Hop, immunophilins, such as cyclophilin (CyP)-40 and FKBP51 are able to bind to hsp90 and following the detachment of hsp70, GR's LBD is open to high affinity hormone binding [220-225]. Owing to their hydrophobic nature and small size, GCs can freely cross the cell membrane. After GC

binding, GR can undergo phosphorylation by several kinases, such as CDKs, GSK3β or MAPKs, and this modification can positively or negatively influence GR activity, i.e. depending on the phosphorylation site, GR nuclear translocation might be enhanced or inhibited. Ligand-bound GR maintains an interaction with the chaperone complex, which facilitates the receptor's translocation along the microtubules toward the nuclear pore complex (NPC). Immunophilins were proven to play an important role in GR translocation by means of their interaction with a motor protein dynein, which moves along the microtubules in an ATP-dependent manner [220,226,227]. Importins 7, 8 and α/β recognize and bind the nuclear localization signals (NLS1 and/or NLS2) within the GR protein and are able to translocate GR through the NPC. In the nucleus, importins release the cargo protein and are shuttled back to the cytoplasm [228,229]. GR nuclear export is facilitated by dissociation of GR from the DNA and is regulated by the nuclear export receptor, calreticulin, which binds to GR's nuclear export signal (NES) and mediates the translocation of the cargo protein through a NPC, back into the cytoplasm. Most probably GR can be alternatively exported via the exportin-1 in a similar manner [220,230].

2.3.2. Genomic actions: transactivation and transrepression

The classical outcomes of the GC-GR signaling pathway are genomic actions, resulting in GR-mediated activation or inhibition of transcription of target genes – mechanisms known as transactivation and transrepression, respectively.

GC-bound GR translocated to the nucleus can dimerize and, as a homodimer, it binds to the DNA at the glucocorticoid responsive elements (GREs), which are imperfect palindrome sequences (5' GGTACAnnnTGTTCT 3'), found in the promoters or within intron regions of GR's target genes [188,231]. GR binding to the GREs attracts chromatin remodeling complexes and co-regulators which facilitate actions of the RNA polymerase II complex [232,233] (Figure 13A). Glucocorticoid-induced leucine zipper (GILZ), serum glucocorticoid regulated kinase (SGK)-1 and mitogen-activated protein kinase phosphatase (MKP)-1, also known as dual-specificity phosphatase (DUSP)-1, belong to factors upregulated by GR via the above GRE-dependent mechanism [234-236]. GR's transcriptional activity can be altered by various transcriptional co-factors (co-activators, co-repressors, co-modulators), of which the functions are associated with remodeling of chromatin or recruitment of the basal transcriptional machinery (BTM). Among GR's co-repressors, interacting with the AF2 region of the antagonist-bound receptor, are nuclear receptor co-repressor (NCoR) and silencing mediator of retinoid and thyroid hormone receptor (SMRT), which repress the receptor's transcriptional activity via the recruitment of histone deacetylases (HDAC) into the transcriptional complex [237-239]. HDACs are enzymes that remove the acetyl groups from conserved lysine residues on histones, contributing to the chromatin's condensation and subsequent gene silencing. Histone acetyltransferases (HATs) reverse actions of

HDACs through acetylation of the histone's lysine residues, leading to an increase in gene transcription [240]. Co-activator binding to the AF2 domain in GR is ligand-dependent. Following agonist binding, the GR conformation changes, which is necessary for the interaction with co-activators, such as members of the p160 family (steroid receptor co-activators, SRCs), cAMP response element-binding (CREB)-binding protein (CBP) or mediator complex subunit (MED)-1 [239,241]. SRCs enhance steroid receptor-mediated transcription by a recruitment of other co-factors, such as CBP holding HAT activity [242]. Alternatively, co-activator binding to the AF1 domain, such as by the SRCs and MED-14, occurs independently of the ligand's presence [243].

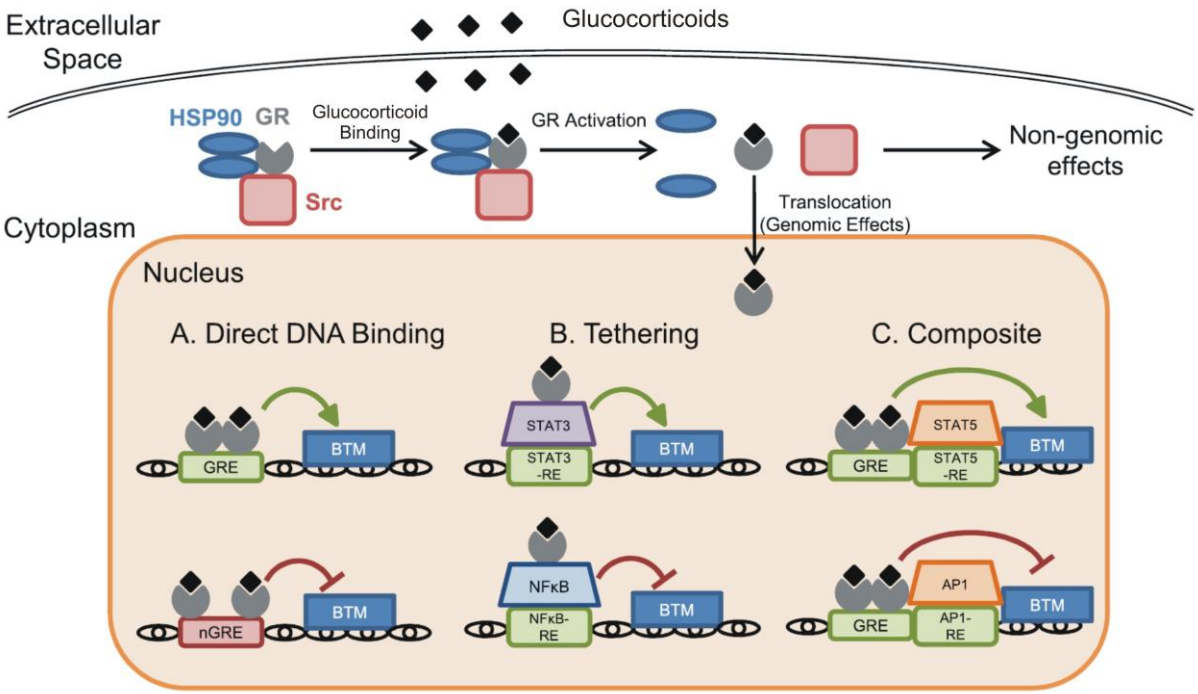

Figure 13. Schematic representation of GR's genomic actions. A) Direct GR binding to the GREs or nGREs results in the target gene's respective activation or repression. B) GR tethering to other transcription factors can indirectly stimulate or inhibit transcription. C) During the composite regulation, GR is interacting both with another transcription factor and with the neighboring GREs. Detailed description in the section 2.3.2. Figure adapted from: [233].

Direct binding of activated GR to negative (n)GREs leads to a repression of expression of several genes including thymic stromal lymphopoetin, insulin, insulin receptor, and the GR gene itself. nGREs are also palindromic sequences (5' CTCCn(0-2)GGAGA 3'), yet GR binds to them as two monomers instead of as a homodimer and involves assembly of GR-SMRT/NCoR complex (Figure 13A). Nevertheless, more research is needed to explore the functionality of this type of elements [244]. GR's indirect regulation of transcription may occur via tethering to another transcription factor occupying the DNA, together or without GR's binding to the DNA, termed as "tethering" and "composite regulation", respectively (Figure 13B,C). Since transrepression mechanisms inhibit the transcriptional activity of (among others) two major pro-inflammatory modulators, the

nuclear factor (NF)κB and the activator protein (AP)-1, this kind of regulation forms the main anti-inflammatory feature of the activated GR [245]. Alternatively, the physical interaction between GR and transcription factors from the signal transducer and activator of transcription (STAT) family can stimulate the transcriptional activity of certain target genes, including β-casein, IGF-1 and toll-like receptor 2 (Figure 13B,C) [246,247].

2.3.3. Role of GR modulation in inflammation

Inflammation is a physiological response to a harmful stimulus, such as infection, mechanical irritation or tissue injury, and it is crucial to protect the affected tissue from any further damage. Inadequate inflammation can result in tissue destruction or systemic spreading of the pathogen, subsequently creating a danger to the whole organism. An excessive or persistent inflammation can also lead to the tissue damage and can give rise of inflammatory afflictions, such as allergies, autoimmune diseases and asthma. Regaining control over inflammatory responses is a key to maintain body homeostasis, and GR modulation plays an important role in terminating inflammation when it is no longer needed [188,233,248]. Glucocorticoids form a standard treatment in therapy of multiple inflammatory disorders including rheumatoid arthritis, inflammatory bowel disease, nephrotic syndrome and systemic lupus erythematosus. Moreover, locally administered GCs help against the ophthalmological disorders, allergic conjunctivitis, dermatitis and asthma [236].

At a molecular level, inflammation is induced by pro-inflammatory signals, such as bacterial LPS, viral double-stranded RNA or the endogenous TNFα and IL-1β. Following binding of these mediators to their respective receptors, the transcription factors (mainly AP-1 and NFκB) are activated resulting in transcription of target genes encoding for pro-inflammatory chemokines, cytokines, enzymes and adhesion molecules [188].

The best characterized pathway of NFκB activation involves binding of TNFα to the TNF receptor (TNFR)-1, which homodimerizes and links the TNFR-associated death domain (TRADD) with the cytoplasmic TNFR-1 death domain. Next, several other proteins are recruited to this complex, including the TGFβ-activated kinase (TAK)-1, NFκB-inducing kinase (NIK) and MAPK kinase kinase (MEKK3), which phosphorylate the inhibitor (I)κBα kinase (IKK) complex, causing its dissociation. Then, IKK phosphorylates IκBα, leading to the inhibitor's further degradation, subsequent release and nuclear translocation of NFκB that binds to the respective gene promoters to stimulate the expression of pro-inflammatory mediators [188].

The main immunosuppressive and anti-inflammatory properties of GCs stem from the GR-transrepressive mechanisms, in which GR in its monomeric form can interact with DNA-bound transcription factor. GR can inhibit actions of multiple transcription factors, including the most studied NFκB and AP-1, but also CREB, nuclear factor of activated T cells (NFAT), interferon

regulatory factor 3 (IRF3), STAT and GATA-3. However, GR-transactivation has also been acknowledged for its role in the suppression of inflammation; GR-driven stimulation of transcription of mediators such as IκBα, MKP-1, lipocortin and GILZ, indirectly affects the inflammatory responses at various stages (Table 4) [248].

Table 4. Examples of inflammatory mediators affected by GR transrepression and transactivation. The anti-inflammatory actions of GR comprise of GR-transrepression, resulting in repression of transcription of multiple pro-inflammatory genes, and of GR-transactivation, resulting in the stimulation of expression of factors with immunosuppressive properties. Source: [248] and [246].

Factors downregulated via GR-transrepression	Factors upregulated via GR-transactivation
cyclooxygenase (COX)-2	annexin A1
E-selectin	dexras
IL-1β	docking protein (DOK)-1
IL-4	GC-induced leucine zipper (GILZ)
IL-5	IL-10
IL-6	inhibitor (I)κBα
IL-8	lipocortin-1
IL-12	MKP-1 (DUSP-1)
inducible NO synthase (iNOS)	p11/calpactin-binding protein
intercellular adhesion molecule (ICAM)	secretory leukoprotease inhibitor (SLPI)
interferon-γ	tristetraprolin (TTP)
monocyte chemoattractant protein 1 (MCP-1)	type II IL-1 receptor
RANTES (CCL5)	
TNFα	
vascular cell adhesion molecule (VCAM)-1	

2.3.4. Non-genomic actions of glucocorticoids

It has been well documented that, apart from classical genomic actions, GCs can induce very rapid (within minutes or seconds) actions without involvement of *de novo* gene transcription. These actions can occur via cytosolic or membrane-associated GR, but interestingly also GC-mediated effects without binding to the receptor are reported [249,250]. The best described non-genomic effect of GCs, driven by the cytosolic GR, involves activation of the endothelial nitric oxide synthase (eNOS) via GR-mediated stimulation of the PI3K/Akt signaling cascade. Phosphorylation and subsequent activation of eNOS results in the production of nitric oxide (NO), which was shown to protect against ischemia-reperfusion injury of the heart in a mouse model [251]. Moreover, due to their lipophilic structure, GCs alone can interact with biological membranes and affect their physicochemical properties. It is suggested that GCs can intercalate plasma and mitochondrial membranes and interact with membrane-associated proteins influencing membrane permeability and lipid peroxidation [252]. In immune cells, these GC-mediated effects result in a rapid reduction of sodium and calcium transport across the plasma membrane, and as

such lead to an impaired lymphocyte energy metabolism and subsequent contribution to an immunosuppressive effect. Moreover, GCs' interactions with the mitochondrial membranes enhance proton leakage causing a diminished ATP production. This can lead to an impaired cytokine production, cell migration and limitation of other important functions of the immune cells, which also limits the immune response [249,253,254].

2.4. Selective GR agonists and modulators

The occurrence of adverse effects associated with a long-term GC use motivated researchers to look for alternative solutions. The ideal compound would hold potent anti-inflammatory properties of GCs and would exhibit a strongly reduced side effect profile. The search for such dissociated compounds is based on the simplified hypothesis stating that most of the anti-inflammatory properties of GCs stem from GR transrepression and that undesirable side effects are associated with the GR transactivation mechanism. In recent years, it has become clear that the reality is far more complex, leading to different strategies [255].

2.4.1. Dissociated glucocorticoids

Synthesis of dissociated GCs or the selective GR agonists (SEGRA) was the first attempt to shift the balance of GC actions toward beneficial anti-inflammatory effects and away from adverse effects, by favoring transrepression over transactivation. Steroidal RU-compounds, RU-24782, RU-24858 (Figure 14) and RU-40066, displayed a significantly reduced transactivation profile (9-35% activity of Dex) with a maintained transrepression activity (58-83% of Dex). RU-24782 and RU-24858 were reported to repress *in vitro* AP-1-dependent reporter genes, IL-1β, MMP-9, and tPA, and displayed the anti-inflammatory effects in the *in vivo* croton oil-induced ear edema mouse model. Unfortunately, as identified later, treatment with the most promising RU-24858 in rats still triggered some of the typical GC-associated adverse effects, including a reduction of bone density [256-258].

2.4.2. Selective GR modulators

Further research led to the synthesis of compounds with a non-steroidal structure, which may or may not bind the classic GR LBD, classified as selective GR modulators (SEGRM) (Figure 14) [256].

A compound with a structure based on a benzopyrano[3,4-f]quinoline precursor, AL-438, was able to preferably bind to GR over other steroid hormone receptors and displayed anti-inflammatory properties both *in vitro* and *in vivo*, by inhibiting the expression of TNFα- and IL-1β-induced IL-6 and E-selectin via the transrepression mechanisms. Moreover, AL-438 not only had a decreased side effect profile in terms of inducing diabetes, as shown in a rat model [259], but also affected chondrocytes *in vitro* to a lesser extent than the classic GCs prednisolone and Dex, suggesting a reduced ability to trigger osteoporosis [260]. AL-438's binding to the receptor caused an alternative protein conformation, resulting in differences in the co-factor-receptor binding profile, deemed responsible for AL-438's beneficial outcome.

Another non-steroidal GR modulator LGD-5552 ((5Z)-5-[(2-Fluoro-3-methylphenyl)methylene]-2,5-dihydro-10-methoxy-2,2,4-trimethyl-1H-[1]benzopyrano[3,4-f]quinolin-9-ol), combines weak GR-transactivation abilities with a relatively stronger GR-driven transrepression. LGD-5552 can

repress TNFα- and IL-1β-induced pro-inflammatory IL-6, E-selectin and MCP-1 expression, while stimulating anti-inflammatory IL-10 expression. Moreover, LGD-5552 was shown to act as an MR antagonist, suggesting potential decreasing effects on the blood pressure, a well-known side effect associated with classic GC use [261].

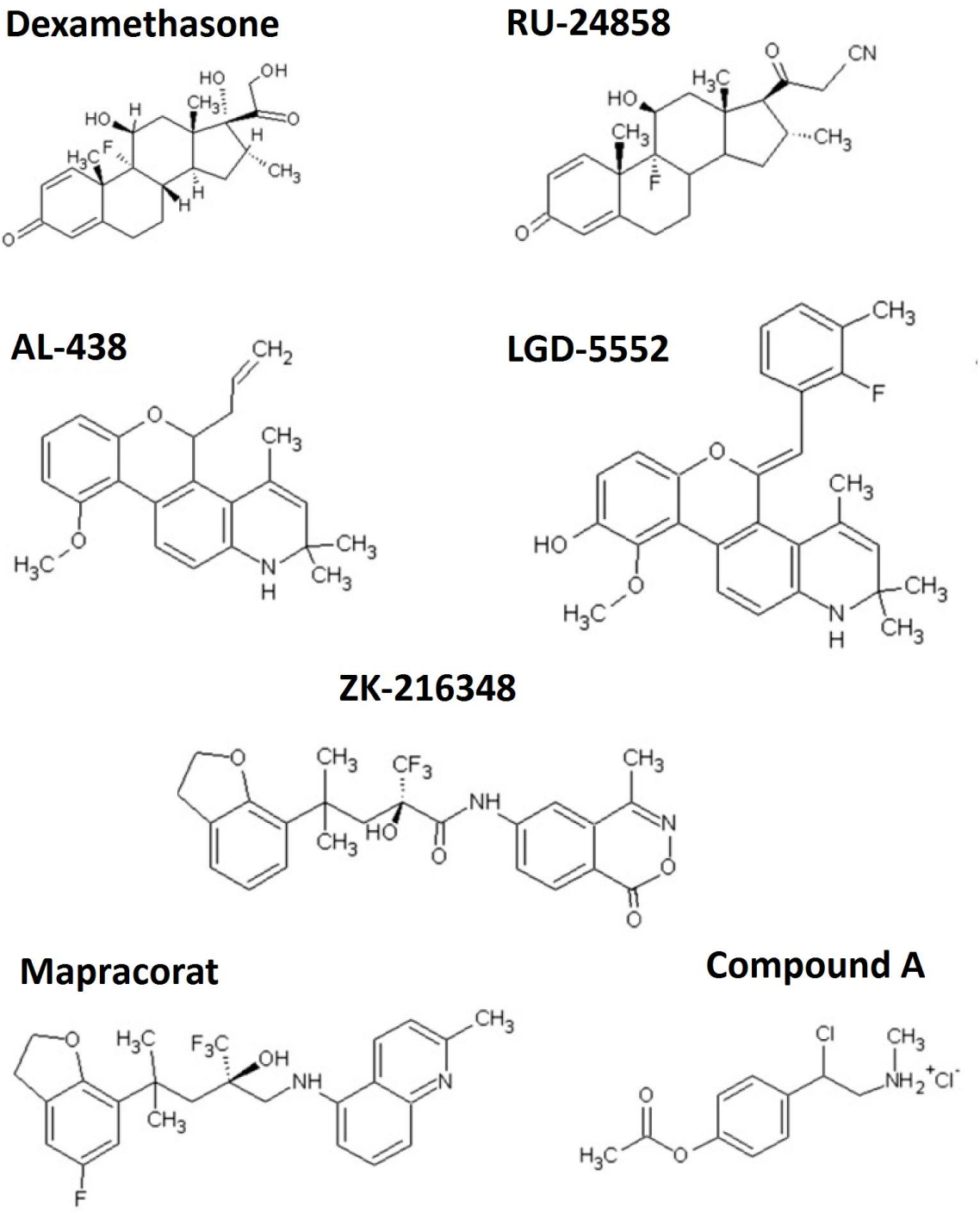

Figure 14. Examples of GR ligands and modulators. Dexamethasone is a classic synthetic glucocorticoid. RU-24858 belongs to dissociated GCs. AL-438, LGD-5552, ZK-216348, Mapracorat and Compound A belong to non-steroidal GR modulators. Description in the section 2.4.1-2. Figure adapted from: [262].

Developed by Bayer Schering Pharma, a non-steroidal compound with a dissociated profile, ZK-216348, showed a promising reduced side effect profile compared to the classic GC Dex. ZK-216348 successfully reduced inflammation in the croton oil-induced ear rat model, did not elevate the blood glucose and displayed diminished skin atrophy when applied topically. Moreover, ZK-216348 displayed no harmful effects on osteoblasts using *in vitro* cultures [263].

Mapracorat (also known as ZK-245186 and BOL-303242-X) reduces inflammation by inhibiting the transcription of several pro-inflammatory cytokines. Recently Mapracorat has completed several phase II clinical trials including the treatment of atopic dermatitis (clinicaltrails.gov identifier: NCT00944632), allergic conjunctivitis (clinicaltrails.gov identifier: NCT01289431) and inflammation following the eye surgery (clinicaltrails.gov identifier: NCT01230125). Mapracorat was shown to reduce inflammation in experimental ocular models and, unlike classic GCs, displayed a less severe side effect profile, including only a minor increase of intraocular pressure. Mapracorat proved to be efficient at preventing early and late allergic responses at the ocular level, which was manifested by a reduced eosinophil recruitment and a reduced production of pro-inflammatory chemokines and cytokines [264,265].

Compound A (CpdA), 2-(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammonium chloride, is a plant-derived aziridine precursor, which was discovered in a Namibian shrub *Salsola tuberculatiformis* Botschantzev, after observation of prolonged gestation period in Karakul ewes following *S. tuberculatiformis*-based diet in periods of draught [266]. Experiments showed that CpdA, a still labile yet more stabilized structure, obtained from the extreme labile active plant fraction, could bind corticosteroid-binding globulins in rat plasma. The follow-up studies revealed that CpdA could activate GR in a selective manner [267,268] and similar to classic GCs, CpdA suppressed NFκB-driven gene expression. Yet, CpdA did not trigger GR transactivation mechanisms. Binding of CpdA to GR induces a different protein conformation compared to binding with GCs. This alternative conformation presumably underpins the inability of GR to form homodimers, and therefore, CpdA-activated GR cannot bind classic GREs [268,269]. In rheumatoid arthritis primary synovial fibroblasts, CpdA was able to inhibit translocation of NFκB's p65 subunit into the nucleus, contributing as such to a suppression of NFκB's actions in a dual GR-dependent and GR-independent manner [270]. GR-independent anti-inflammatory properties of CpdA were further investigated in bone marrow-derived dendritic cells, where expression of the pro-inflammatory granulocyte-macrophage colony-stimulating factor (GM-CSF) was suppressed by CpdA in GR-knocked down cells and in case of pharmacological blockage of GR [271]. In osteoarthritis synovial fibroblasts, CpdA in contrast to prednisolone, did not induce leptin production, related to osteoarthritis severity [272]. And moreover, CpdA was shown effective in reducing mRNA and protein levels of GC-resistant chemokines (CCL5, CX3CL1 and CXCL10) in airway smooth muscle

cells, suggesting its potential beneficial role in treatment of GC-resistant asthma [273]. Adding to its immunosuppressive benefits, in analogy with the effect of cells under heat shock conditions, CpdA was shown to stimulate the expression of the GR chaperone hsp70, in a GR-dependent manner. Elevated intracellular hsp70 levels exert anti-inflammatory effects via obstructing the inhibitor (I)κB degradation and a subsequent impaired NFκB p65 translocation [274]. The anti-inflammatory properties of CpdA were confirmed in animal models, including acute zymosan-induced arthritis and experimental autoimmune encephalomyelitis models, and were accompanied by less adverse effects than classic GCs, including absence of hyperinsulinemia and hyperglycemia [268,275].

2.5. Glucocorticoid receptor modulation in health and disease

GC-driven actions exert pleiotropic effects on the organism (Table 5). The endogenous GCs play key roles in many fundamental processes involving embryonic development, function of the nervous, cardiovascular, immune and respiratory systems, glucose and lipid metabolism and the reproduction [195]. Synthetic GCs are used in the therapy of many inflammatory disorders, including asthma, chronic obstructive pulmonary disease (COPD), inflammatory bowel disease, and various allergies. Moreover, GCs are applied as immunosuppressors in order to minimize the risk of a transplant rejection. GCs are often co-administered during chemotherapy of various solid tumors, pre- as well as post-surgery. Because of their pro-apoptotic properties in lymphocytes, GCs are part of the therapy against hematological malignancies. And furthermore, GCs angiostatic properties are beneficial in the treatment of several angiogenic afflictions, such as infantile hemangiomas [195,276-278].

2.5.1. Glucocorticoid therapy in the hematological malignancies

Owing to their ability to trigger apoptosis in hematological cells, GCs are used as chemotherapeutic agents for the treatment of acute lymphoblastic leukemia, chronic lymphoblastic leukemia, multiple myeloma, Hodgkin's and non-Hodgkin's lymphomas. GC-induced apoptosis involves transactivation activity of GR and activation of the caspase cascade, which most probably occurs via the intrinsic mitochondrial-mediated pathway. This intrinsic pathway involves destruction of the mitochondrial membrane by Bim, Bax and Bak, pro-apoptotic proteins from the Bcl-2 family. This results in a release of cytochrome c and apoptotic peptidase-activating factor-1, leading to further activation of caspase 9 and subsequent downstream effector caspases 3, 7 and 6. Unfortunately, the prolonged GC use, is associated not only with the emergence of side effects, but also with the occurrence of GC resistance in the clonal lymphocyte populations, a factor that strongly limits GC chemotherapy. GC resistance in lymphocytes can result from multiple events such as homologous down-regulation of GR gene, mutations of GR or deregulation of Bcl-2 family members' functions [277,279]. The phenomenon of GC resistance is described in section 2.2.4.

2.5.2. Effects of GR modulation in solid tumors

Glucocorticoids are frequently used in the therapy of solid tumors (including brain, prostate, breast, lung and colon cancer), in the combination with surgery, radio-, chemo- or hormonal therapy. Because of their anti-emetic and anti-edemic properties, GCs have been administered to reduce the symptoms of the disease, to reduce side effects of chemotherapy and in order to protect healthy tissues from cytotoxic effects of the treatment. Although the administration of GCs has many benefits in the solid tumor treatment, it has been also associated with undesirable

effects, such as induction of chemotherapy resistance and protection from apoptosis, strongly contrasting with the GC-mediated effects in hematological malignancies. Nevertheless, depending on the cancer type the benefits vs. drawbacks of GCs will need to be considered in a case-specific manner (Figure 15) [278,280-282].

Table 5. Roles of GCs in major human organ systems. Glucocorticoids affect the function of almost all organ systems, their beneficial effects are used in clinic, in the therapy of various diseases, however, the occurrence of adverse effects often limits the use of GCs. Source [195] and [231].

Organ system	Role of the endogenous GCs	Beneficial use of synthetic GCs	Adverse effects of GCs
Nervous	Physiological homeostasis Response to stress		Psychiatric disorders
Cardiovascular	Anti-inflammatory Angiostatic Cardiomyocyte survival Blood pressure and vascular tone homeostasis		Cardiovascular disease
Immune	Suppression of expression of pro-inflammatory cytokines Regulation of the immune cell maturation, migration and apoptosis	Organ transplant	Activation of latent viruses Susceptibility to infections
Musculoskeletal	Anti-inflammatory Muscle metabolism Insulin resistance Osteoblast apoptosis Osteoclastogenesis	Rheumatoid arthritis	Osteoporosis Muscle atrophy Growth retardation
Visual	Anti-inflammatory Angiostatic Photoreceptor survival	Uveitis Keratitis Diabetic retinopathy	Glaucoma Cataract formation
Respiratory	Suppression of expression of cytokines, chemokines and cell adhesion molecules	Asthma COPD	
Reproductive	Regulation of gonadal function Embryonic organ development	Lung maturation at preterm birth	Gonadal virilization
Integumentary	Anti-inflammatory Regulation of epithelial integrity	Psoriasis Dermatitis	Impaired wound healing Skin atrophy
Glucose and lipid metabolism	Glucose level regulation Lipid homeostasis		Centripetal obesity Hyperglycemia Dyslipidemia

2.5.2.1. Beneficial effects of GCs in cancer

Prednisolone, hydrocortisone and dexamethasone, for their ability to suppress adrenal androgen production, are often used as a secondary hormonal treatment for patients with castration-resistant prostate cancer (CRPC). Moreover, prednisolone forms a part of chemotherapy strategy for metastatic CRPC, as it is proven effective in combination with abiraterone, docetaxel, and cabazitaxel [283,284]. Many *in vitro* studies have shown the beneficial role of GC administration against androgen-independent prostate cancer cells. Dex suppresses the activity of NFκB and expression of IL-6 resulting in growth inhibition of DU145 and PC3 prostate cancer cell lines [285,286]. Moreover, GCs were proven to have anti-angiogenic effects by repressing VEGF and IL-8 production by prostate cancer cells, in concordance with *in vivo* results, showing a reduced tumor growth, vasculature and expression of VEGF and IL-8 in the xenograft mouse model [287].

Budesonide, a synthetic GC used for treatment of asthma, displayed chemopreventive properties when administered as an aerosol into lungs of mice that were exposed to a carcinogen benzo(*a*)pyren. Budesonide reduced the tumor load by 78% compared to the control group. Moreover, *in vitro* studies on lung cancer cells A549 and H1299 showed growth-inhibitory effects of budesonide in a dose- and time-dependent manner [288].

GCs have been used in the therapy of brain cancer for decades, mainly due to their ability to minimize cerebral edema and risk of a radiation-associated encephalopathy, however, the exact mechanism behind GC's anti-edemic properties is not clear [289]. Dex was shown to have proliferation-inhibitory properties in several human glioma cell lines [290]. Moreover, Dex displayed anti-invasive properties in U87GM malignant glioma cells, in a MKP-1-dependent mechanism. GR-driven upregulation of MKP-1 led to reduced expression and activity of matrix metalloproteinase (MMP)-2, an extracellular matrix protein implicated in cell invasion. Similar anti-invasive properties of Dex were observed in an *in vivo* chick chorioallantoic membrane (CAM) model with U87GM cells [291].

GCs' ability to inhibit proliferation in the estrogen-regulated ER-positive breast cancer cells was previously described in several studies, while this effect was not detected in the ER-negative breast cancer cells [292,293]. As for the underlying mechanism, Dex was shown to inhibit estrogen-stimulated ER-driven expression of target genes. In cells treated with both Dex and estrogen, chromatin immunoprecipitation (ChIP) analysis revealed GR binding to the estrogen-binding regions (EBRs), which blocked transcriptional activity of the ER. Interestingly, the treatment with Dex or estrogen alone did not result in occupation of the same EBRs by the GR [294].

In a study conducted on the ER-negative breast cancer cells MDA-MB-231, GC treatment resulted in a morphological alteration and reduced cell invasion. The mechanism behind this loss of

aggressiveness involved a GR-mediated induction of CCN5 expression. CCN5, a factor belonging to the CCN family (connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed) is involved in multiple functional cascades including cell survival, migration, mitogenesis, cellular adhesion and angiogenesis [295]. CCN5 was shown to repress genes associated with EMT, leading to a subsequent downregulation of mesenchymal markers, such as vimentin, cadherin 11 and ZEB1 [296].

The anti-invasive properties of Dex were also observed in bladder cancer cells, where GC treatment down-regulated MMP-2, MMP-9, IL-6 and VEGF and induced mesenchymal-to-epithelial transition. However, in the same study Dex stimulated cancer cell growth by protecting the cells from apoptosis in conditions with and without cisplatin administration. In the *in vivo* mouse xenograft model, animals treated with Dex exhibited slightly larger tumors, but were spared from development of bloody ascites, associated with cancer cell dissemination and the formation of metastasis [297].

GCs were recently described as agents able to inhibit ovarian cancer metastasis by inducing expression of microRNA (miR)-708 and a subsequent repression of Rap1B. Low miR-708 and high RapB1 levels are detected in late stages of ovarian cancer patients and are associated with a poor prognosis. GC-driven downregulation of Rap1B caused inhibition of migration and invasion of ovarian cancer cells and reduced formation of metastasis in the orthotopic xenograft mouse model. The anti-invasive effect was reverted by restoration of the Rap1B expression [298].

2.5.2.2. Undesirable effects of GCs in cancer

Nevertheless, many recent studies have reported detrimental effects of GR activation in terms of induction of chemoresistance in cancer cells [280]. A large study which involved tests on primary and commercial cancer cell lines of different origin, demonstrated protective properties of GCs against diverse cytotoxic therapies. Tested treatments included cisplatin, etoposide, γ-radiation, gemcitabine, methotrexate, 5-FU, cytarabine and paclitaxel. Dex, prednisone, betamethasone and hydrocortisone, all displayed similar therapy resistance-inducing properties and the mechanism of this resistance originated from a GC-driven inhibition of apoptosis [299]. GR-activation was associated with a direct expression of SGK-1, MKP-1 and IκB, required for the GR-mediated protection from cell death [300,301]. Furthermore, a recent study on prostate cancer has reported that GR expression is associated with resistance to enzalutamide, a drug that targets the androgen receptor signaling. GR, by substituting for the AR blocked by enzalutamide, activated a similar set of target genes and led to the maintenance of a resistance phenotype. In a preclinical model, as well as in patients' samples, the administration of Dex conferred to chemoresistance, and this effect was reversed with use of a GR antagonist, compound 15 [302]. Similar observations were made in breast and ovarian cancer. Pre-treatment with Dex significantly

inhibited therapeutic effects of paclitaxel in breast and ovarian xenograft tumors [303]. Furthermore, a treatment with Dex inhibited cisplatin- and gemcitabine-induced apoptosis *in vitro* in multiple established pancreatic cancer cell lines, as well as in primary cell lines obtained from the resected pancreatic tumors, which was also confirmed in an *in vivo* xenograft model [304]. Moreover, another study has reported proliferation-stimulatory actions of GCs, observed in multiple different cell lines including breast and lung carcinomas, as well as in cell lines of mesenchymal and neuroectodermal origin [305].

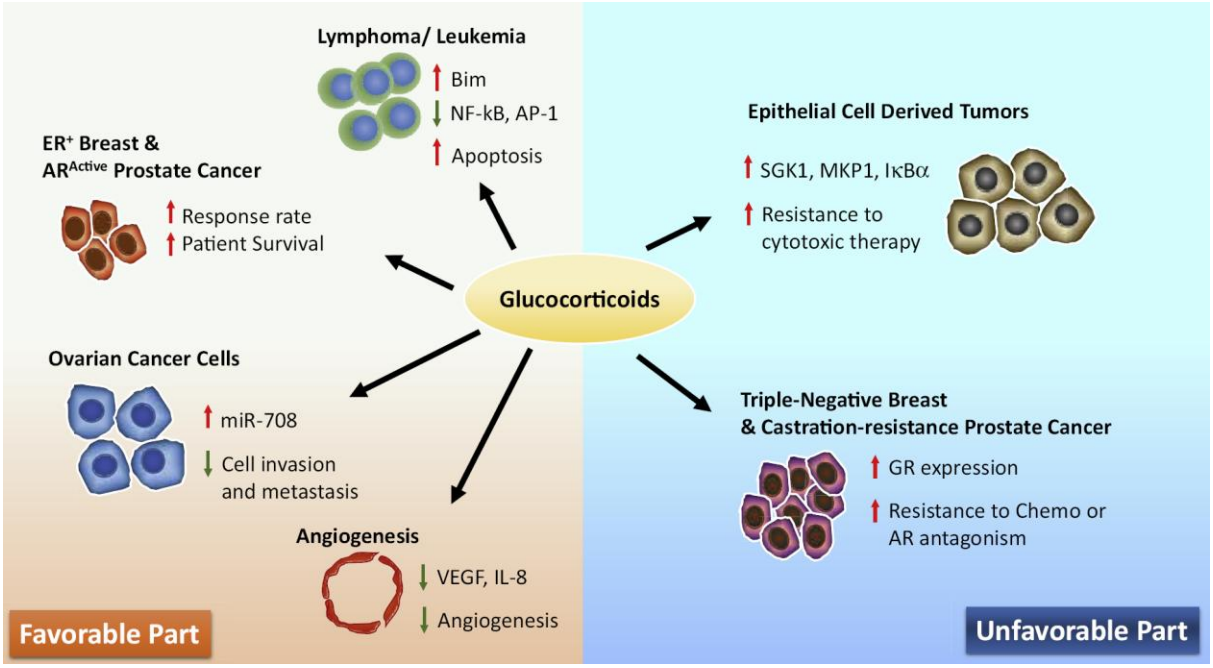

Figure 15. Current understanding of GC-mediated effects in cancer. Figure source [282].

2.5.2.3. Effects of GCs in colorectal cancer

Similarly to other solid tumors, GC administration in CRC was reported to have both beneficial and detrimental effects. Although it was earlier suggested as possible cause of colon carcinogenesis, a frequent systemic use of GCs was shown not to increase the risk of CRC, as reported in a population-based case-study in Northern Denmark [306]. GCs (such as Dex or methylprednisolone) given to patients prior CRC surgery, were shown to significantly reduce inflammation, cardiopulmonary stress and postoperative pain [307]. Nevertheless, a preoperative Dex administration was associated with a higher rate of cancer recurrence, compared to a placebo-treated group, as reported in a 5 year follow-up analysis. However, the authors highlighted the relatively small sample size (43 patients,) and therefore, they appeal to interpret their results with caution [308]. *In vitro*, Dex was shown to inhibit a TGF-β-induced EMT and cell migration of colon cancer cells. Dex inhibited the ERK and Akt signaling pathways, leading to a decrease in cystein-rich angiogenic inducer (CYR)61 expression, a factor implicated in the

promotion of cancer proliferation and invasion [309]. Furthermore, Dex was shown to inhibit a hypoxia-induced EMT. Dex treatment of colon cancer cells *in vitro* under hypoxic conditions resulted in a downregulation of HIF-1α, VEGF, and several EMT-related genes (encoding for transcription factors Snail, Slug and Twist). Moreover, induction with Dex inhibited colon cancer cell migration and invasion and furthermore recovered E-cadherin levels and a morphotype characteristic for the epithelial cells grown under normoxia [310]. Nevertheless, GC-driven protection against chemotherapy was reported in a study conducted on several colon and rectal cancer cell lines, as well as on cultures obtained from the resected tumors. Dex prevented cisplatin-induced apoptosis and promoted cell proliferation in the tested established cell lines, as well as in the surgical specimens of colorectal carcinomas, correspondingly to the *in vivo* xenograft model results, where Dex also induced chemotherapy resistance [311]. Another study suggested that GCs promoted formation of metastases of colon cancer cells injected into the murine spleen. A detailed analysis revealed that the injected cells, pretreated with Dex, were partially protected from a serum-mediated cytotoxicity, which enabled them to survive in the blood stream with higher chances of forming micrometastases. Interestingly, colon cancer cells treated with Dex, unlike the untreated control, were able to proliferate in pure serum [282].

The level of GR expression in colorectal cancer cells is also an interesting aspect, noting that an immunohistochemical examination of 91 cases of human colon cancer revealed that 48% of analyzed tumors were GR-positive, independently of patient's age, gender or survival. GR expression, however, correlated with the levels of cell-cycle related molecules: retinoblastoma protein (pRb) and p16, belonging to group of tumor suppressors, in view of their function in cell cycle arrest [312]. Moreover, as another study reports, in the colorectal carcinoma samples obtained from patients, GR mRNA expression was significantly (approximately 50%) lower compared to the normal colonic mucosa from the corresponding patients, and interestingly, GR mRNA levels negatively correlated with E-cadherin mRNA levels [313].

2.5.2.4. GR-modulation and cancer stroma

Due to their lipophilic structure, systematically administered GCs affect all the cells in the body, with the emphasis on cells expressing a functional GR protein [174]. Stromal cells play an unquestionable role in cancer progression, therefore, there are research studies focusing on the stroma as a target or tool for cancer therapy [167].

Cancer was described as "wound that does not heal", because the process of tumor progression resembles mechanisms occurring during wound healing [69]. Wound healing involves cooperation of various cell types, including fibroblasts and myofibroblasts, endothelial cells, and inflammatory cells. Wound healing is a controlled process composed of three overlapping phases: inflammation, proliferation and maturation (tissue regeneration) [314]. Glucocorticoid treatment substantially

affects all phases due to suppression of inflammation and angiogenesis, decrease in collagen production, effects on cell metabolism and inhibition of expression of key factors involved in the wound healing process [315-317]. Subsequently, GC treatment can lead to skin atrophy [195]. GCs' impact on dermal fibroblasts might point to a potential beneficial outcome of the treatment during cancer therapy (Table 6).

Table 6. Effects of glucocorticoid treatment on dermal fibroblast and potential implications to cancer

Dermal fibroblasts	Glucocorticoid-mediated actions	Potential relation to cancer	Reference
Cell proliferation	Controversial data on the impact of GCs on dermal fibroblast proliferation In keloid fibroblasts, triamcinolone delays fibroblast growth	Potential inhibition of CAF proliferation	[318,319]
Cell migration	GC treatment inhibited cell migration	Decreased cell invasion	[320,321]
Expression of pro-inflammatory genes	GCs suppress production of pro-inflammatory factors, including IL-6, IL-8, and GM-CSF	Suppression of cancer-associated inflammation Reduced stimulation of macrophage infiltration	[320,322]
Collagen production and metabolism	GCs decrease expression of collagen type I and III, collagenases, TIMP-1, and TIMP-2	GC-mediated interference with collagen metabolism, possible reduction of tumor stiffness, but also facilitated cell migration	[320,323]
TGFβ expression	GCs decrease TGF-β1 synthesis	Possible decreased cancer aggressiveness, delayed cancer progression	[324]
Hyaluronic acid production	GC-mediated decrease of hyaluronan production	Potential decrease of hyaluronan-linked increased cancer progression, angiogenesis and metastasis	[325,326]
Fibrosis and abnormal scar formation	Corticosteroid treatment is effective in treating keloid and hypertrophic scars Potential GC anti-fibrotic effects due to suppression of inflammation in cystic fibrosis model	Potential reduction of fibrosis formation Chronic fibrosis can predispose tissue to develop Cancer cancer progression is often accompanied by fibrosis	[327-329]

Nevertheless, the role of GR modulation in a tumor stroma is still poorly understood. A recent study showed that the majority (91%) of 56 tested breast cancer samples contained GR-positive peritumoral myofibroblasts. Interestingly, GR expression positively correlated with the tumor

grade and with GR expression in the epithelial component [330]. In the study on prostate cancer microenvironment, a functional GR was detected in myofibroblasts derived from cancer-associated stroma (CAS), as well as in cells from benign-associated stroma (BAS). Intriguingly, GR-mediated transcriptional activity varied between CAS-derived cultures, appearing higher or lower compared to BAS-originated cells [331].

2.5.3. Angiostatic properties of GCs in cancer

GCs' angiostatic properties are well-documented and acknowledged in many therapies, including the treatment of diabetic retinopathy and infantile hemangioma [276,332-334]. Moreover, GCs were observed to reduce vasculature in several malignant tumors. Although the mechanism behind the angiostatic properties of GCs is not completely clear, the GR-mediated inhibition of VEGF expression in cancer cells was reported in several cases, including prostate cancer, head and neck carcinoma, Lewis mice lung carcinoma and renal cell carcinoma, of which the latter belongs to highly vascularized tumors, due to overproduction of pro-angiogenic factors, independently of the oxygen concentration [287,335-338]. In glioma cell lines Dex triggered apoptosis, but also appeared to elevate VEGF levels in a concentration-dependent manner. However, in an *ex vivo* assay Dex reduced brain tumor vascular density, showing its overall angiostatic character [339]. In a recent study on hepatocellular carcinoma Dex was shown to reduce tumor growth and to inhibit the angiogenesis. Dex treatment increased expression of genes regulating gluconeogenesis in the hepatocellular cancer cells, and the conditioned medium collected from such cultures inhibited the migration, tube formation and permeability of model human umbilical vein endothelial cells (HUVECs) [340]. Dex was also shown to reduce the TAM-induced expression of pro-inflammatory and pro-angiogenic factors IL-8, IL-6 and RANTES in the glioma cells. These results suggested Dex's therapeutic role, as the analysis of glioma specimens obtained from patients revealed positive correlation between TAMs count, IL-8 expression and the microvessel density [341]. Similar observations were previously made in a case of human lung adenocarcinomas [342]. Furthermore, Dex was proven to enhance anti-angiogenic effects of Endostar, a recombinant human endostatin, as shown in the *in vitro* assays with use of HUVECs, as well as in an *ex vivo* rat aortic assay and *in vivo* chicken CAM assay. Moreover, in a mouse xenograft model, Endostar administered with Dex reduced tumor growth to a higher extent than when drugs were applied separately [343]. Furthermore, detailed studies on the endothelial cell (EC) response to GCs revealed that the direct angiostatic properties of GCs originate from the reduction of ECs' ability to form tubular structures. This inhibition was shown to be a result of changes in the cell morphology and not cell viability, migration or proliferation. Mechanistically, these GR-driven changes are suggested to be partially mediated by the induction of thrombospondin-1 expression, a potent endogenous angiostatic factor [344].

PART II: RESEARCH OBJECTIVES

Glucocorticoids (GCs) are prescribed for cancer patients pre- and/or post-surgery and during chemotherapy. However, the impact of these GCs on solid tumor biology is controversial and not fully understood [278,281]. The glucocorticoid receptor (GR) is expressed in almost every human tissue. Therefore, a systemic GC use affects virtually all cells within the body. Nevertheless, the analysis of specimens obtained from colorectal cancer patients revealed that approximately 50% of these tumors are GR-negative [312]. Obviously, the GR expression profile of cancer cells will significantly impact the outcome of a GC treatment. Cancer progression is further supported by surrounding stromal cells, such as CAFs [76]. Although the CAFs' role in cancer is well recognized, the impact of GC treatment on the tumor biology and angiogenesis via indirect effects on CAFs is yet unknown. Moreover, given the occurrence of many adverse effects associated with GC treatment, alternative GR stimulants are being studied, including the selective GR modulators (SEGRM), such as a plant-derived compound A (CpdA) [256]. The impact of CpdA on CAFs, endothelial cells, and on angiogenesis has never been described.

Given this lack of information, the aim of this doctoral dissertation was to answer the following research questions:

1. What is the impact of GCs and an SEGRM CpdA on CAF biology?
2. How do CAF-derived factors affect endothelial cells and the process of angiogenesis and what is the impact of GR-mediated changes in the CAF-derived secretome on angiogenesis and endothelial cells?
3. How do GR-mediated changes in CAFs affect their pro-stimulatory influence on GR-deficient colorectal cancer cells?
4. What is the impact of the GC dexamethasone and the SEGRM CpdA on endothelial cell behavior and angiogenesis?

The **Part III: Results** section of this dissertation aims to answer the above questions in four corresponding chapters (articles) (Figure 16). **Article 1** comprises a study on GCs- and CpdA-derived effects on different aspects of CAF biology, in which the analysis of the cells' responsivity to treatment reveals important GR-mediated changes in the CAF secretome. **Article 2** presents results on an analysis of effects of CAF-derived culture medium on endothelial cells and angiogenesis, and moreover, it shows how the treatment with GCs affects this impact of CAFs. Furthermore, in **Article 3** we focus on the influence of GC-treated CAFs and their secretome on the GR-unresponsive colorectal cancer cells. Lastly, in **Article 4** we attempted to explain differences between dexamethasone's and CpdA's direct effects on endothelial cells' behavior and the process of angiogenesis.

Finally, in the last part of this dissertation, the **General Discussion**, the main findings are recapitulated, the meaning of the obtained results is explained, the limitations of the study are revealed, and the future perspectives are discussed.

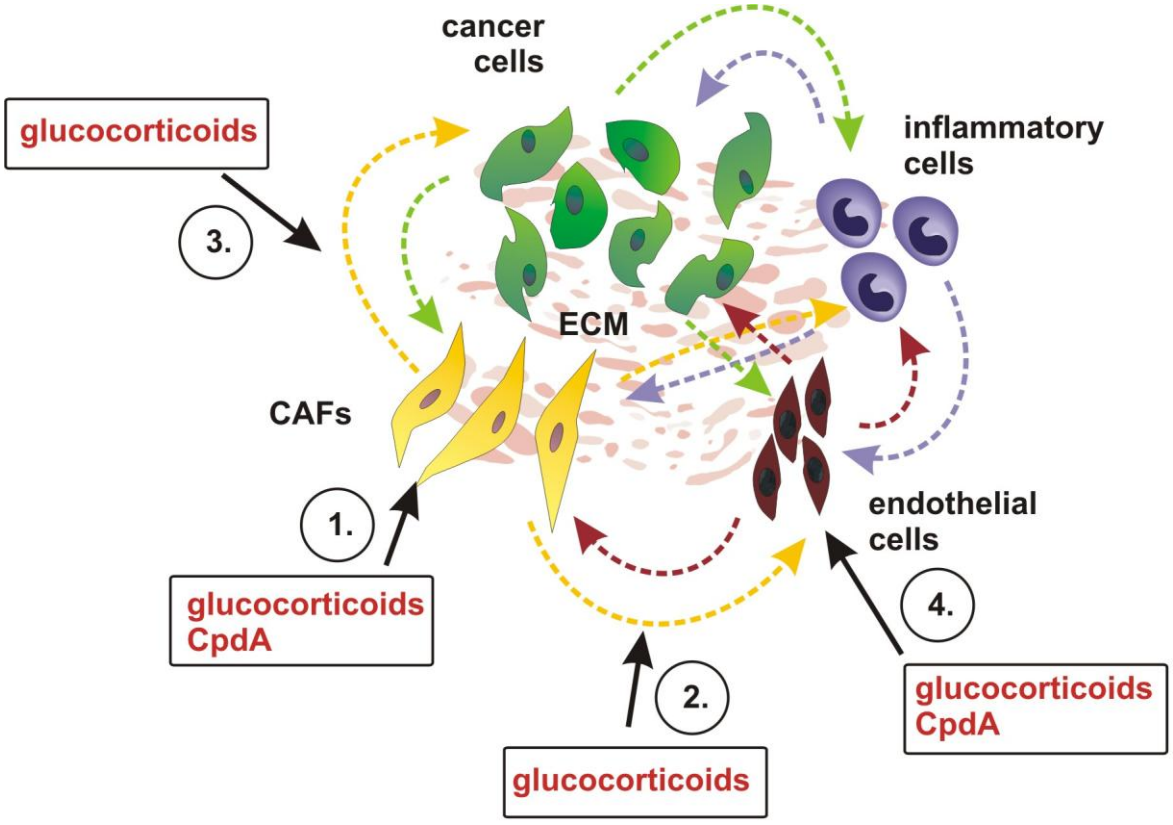

Figure 16. A schematic representation of cross-talk in the tumor microenvironment and the research questions covered in this dissertation. Dotted arrows represent an impact of particular cellular components on the neighboring cell populations; numbers 1-4 indicate the chapter numbers in the Results section answering the corresponding research questions.

PART III: RESULTS

CHAPTER 1: ARTICLE 1.

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Glucocorticoids and the non-steroidal selective glucocorticoid receptor modulator, compound A, differentially affect colon cancer-derived myofibroblasts

Zuzanna Drebert, Marc Bracke, Ilse M. Beck *

Laboratory of Experimental Cancer Research, Department of Radiation Oncology & Experimental Cancer Research, Ghent University, Gent, Belgium

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Abstract

The glucocorticoid receptor functions as a ligand-dependent transcription factor that positively or negatively regulates the transcription of various specific target genes. Not only steroidal glucocorticoids can bind and activate the glucocorticoid receptor, but also the intensively examined non-steroidal selective glucocorticoid receptor modulators can do so, albeit with a select effector profile skewed to glucocorticoid receptor transrepression. Glucocorticoids are widely used to treat inflammatory afflictions, but also as part in anti-cancer therapies or adjuvants thereof. As the impact of glucocorticoids and selective glucocorticoid receptor modulators has scarcely been researched in this setting, we focused on colon cancer and its stromal environment, in particular the stromal myofibroblasts, which are known to influence cancer cells via paracrine signaling. In these myofibroblasts, also known as cancer-associated fibroblasts (CAFs) the glucocorticoid dexamethasone is able to drive the glucocorticoid receptor into the nucleus and thus negatively regulates the expression of particular pro-inflammatory genes in TNFα-stimulated cells. The selective glucocorticoid receptor modulator compound A has an impaired ability to translocate GR, presumably underpinning its modest anti-inflammatory properties in these cells. Only dexamethasone, and not compound A, can upregulate the glucocorticoid receptor transactivation-dependent GILZ expression. Neither dexamethasone, nor compound A affect CAF viability. However, compound A retards the growth of this CAF cell line. Additionally, dexamethasone can inhibit the expression of tenascin C, hepatocyte growth factor, and TGFβ, which are all factors known for their impact on colon cancer cell invasion, in a glucocorticoid receptor-dependent manner. In contrast, compound A can only slightly diminish the expression of just hepatocyte growth factor, and not tenascin C or TGFβ. Combined, our results expose new tumor microenvironment-modulating effects of glucocorticoids and the selective GR modulator compound A.

1.1. Introduction

Hallmarks of cancer development involve sustained proliferative signaling, growth suppressor evasion, replicative immortality, angiogenesis, invasion and metastasis [8]. Some of those processes can be enhanced by adjacent tumor-associated stromal cells and the tumor-associated inflammatory response [8,35]. Many mechanistic questions on the regulation of invasion and metastasis still remain unanswered, despite recent progress.

Colorectal cancer is the third most common cancer in Europe and the USA, and one of the prominent causes of cancer-related deaths [345]. The incidence of morbidity increases with age and is associated with the Western dietary lifestyle. Although there is no single cause for developing colorectal cancer, certain hereditary diseases, such as familial adenomatous polyposis or Lynch syndrome, strongly elevate the risk of disease [38]. The majority of colon cancers start in glands of the intestine lining. The disease progresses from normal mucosa towards hyperplasia and finally carcinoma *in situ*, which ultimately leads to an invasive and metastatic cancer [35,38].

Cancer cell invasion and metastasis are driven by changes occurring in the cancer stroma as a result of the cross-signaling between cancer cells and their surroundings. In this tumor microenvironment, myofibroblasts arise, which share characteristics with smooth-muscle cells and fibroblasts. Myofibroblasts have a spindle-like shape with an indented nucleus, well-developed fibronexus junctions and stress fibers. They can be divided in bone marrow-derived myofibroblasts, which originate from mesenchymal stem cells, and non-bone marrow-derived myofibroblasts. The latter cells can originate from precursors, such as endothelial cells, fibroblasts, smooth muscle cells or non-malignant epithelial or epithelial-derived carcinoma cells. Apart from their role in embryonic development and tissue morphogenesis, myofibroblasts appear in the pathology of fibrosis and cancer development. Cancer cell-derived cytokines, such as transforming growth factor β (TGFβ) and other growth factors promote differentiation of surrounding stromal precursors into myofibroblasts, also known as cancer-associated fibroblasts (CAFs), which in turn modulate cancer invasion through cell-to-cell or paracrine signaling. Among the many factors expressed by CAFs we find chemokines and cytokines, such as IL-6 and RANTES, growth factors, such as TGFβ and hepatocyte growth factor/scatter factor (HGF/SF), as well as proteins involved in matrix remodeling, such as matrix metalloproteinases (MMPs) and tenascin C (TNC) [35,76,346,347].

Glucocorticoids (GCs) are widely used as a treatment strategy against inflammatory and autoimmune diseases [204], but also in anti-cancer therapies or as adjuvants thereof [348]. These steroids can bind and activate the glucocorticoid receptor (GR), which is a member of the superfamily of nuclear receptors and hence also known as NR3C1. Activated GR is then

transported to the nucleus where it can act as an activating or repressing transcription factor. Ligand-bound GR can, among other mechanisms, directly modulate transcription through interaction with glucocorticoid responsive elements (GRE) or indirectly via tethering with another transcription factor, such as NFκB or AP-1. This indirect modulation of transcription, often occurring in transrepression, solidly contributes to the anti-inflammatory properties of GCs [188,245,349].

Recent pharmacological advances have allowed to dissociate the transactivating and repressing mechanisms of GR modulation via the use of selective GR modulators (SEGRMs), which are designed to modulate only GR-mediated gene repression [256]. The SEGRMs were developed based on the hypothesis that the expression of many genes involved in undesirable GC-associated side effects [209] are mainly due to GR transactivation mechanisms, whereas the repression of pro-inflammatory gene expression forms the mainstay of GCs' anti-inflammatory mechanism [255].

In that respect, 2-(4-acetoxyphenyl)-2-chloro-N-methylethylammonium chloride, named compound A (CpdA), a plant-derived precursor of phenyl aziridine, is a non-steroidal SEGRM that is under intensive investigation [255,267,268,350-352]. CpdA differs from classic GCs in size and structure. Nonetheless, CpdA was shown to effectively modulate GR. Unlike GCs, CpdA does not induce GR dimerization and fails to induce an increased GR S211 phosphorylation. Both events are most likely due to a different conformation of CpdA-bound versus GC-bound GR. Concomitantly, CpdA-bound GR is unable to physically bind GRE-driven promoters, culminating in its inability to trigger transactivation of these GRE-driven promoters. *In vivo* evidence shows that CpdA has a more favorable side-effect profile compared to classic GCs [255,268], confirming the rationale to develop and explore these SEGRMs. Moreover, recent studies showed that CpdA has an impact on cancer cells, as it potentially binds to both the androgen receptor and GR in prostate carcinoma, leading to an inhibited proliferation and induction of apoptosis of prostate cancer cells [353]. CpdA treatment also decreases cell proliferation and increases apoptosis of CEM and K562 leukemia cells [351].

Given that GCs bare pro-apoptotic properties, these steroids have been widely used in combination with other treatments in lymphoid malignancies. However, their role in solid tumor biology is still ambiguous. On the one hand, the use of GCs can induce chemoresistance in, among others, prostate and cervical cancer therapy, and through parallel down-regulation of the immune response it might facilitate metastasis [280,354]. On the other hand, in breast cancer cells, GCs are known to indirectly repress epithelial-to-mesenchymal (EMT)-associated gene expression, eventually impacting the invasiveness of these cells [296]. The role of GCs is also contested in bladder cancer, where GCs actually increase cell proliferation and activity, but at the same time

suppress the expression of multiple invasion-associated genes and also cause mesenchymal-to-epithelial transition (MET) [297].

Although GCs are commonly used in the treatment of certain cancers, cancer-related complications and inflammation, the GC- and especially SEGRM-mediated effects on the intricate cancer eco-system still remain largely unknown. As stromal cells affect the invasive progression of cancer cells by modulating cytokines, chemokines and other molecules and as these factors are potentially targeted by GR regulation, we explored the impact of GCs and the SEGRM CpdA on the biology and the particular gene expression pattern of key factors of stromal, colon cancer-derived CAFs.

1.2. Materials and Methods

1.2.1. Cells and reagents

Human stromal colon cancer-derived CAFs (CT5.3hTERT cells) [77], and murine L929sA fibroblasts were cultured in DMEM (Gibco, Life Technologies) supplemented with 10% fetal calf serum (Greiner bio-one), 100 U/ml penicillin and 0,1 mg/ml streptomycin (Gibco, Life Technologies), and were grown at 37°C with either 10% or 5% CO_2, respectively. In all experiments, we used medium supplemented with charcoal-stripped serum (Gibco, Life Technologies).

The classic GC dexamethasone (Dex) was purchased from Sigma-Aldrich and the origin and handling of the SEGRM compound A (CpdA) was described previously [268]. Additionally, we used hydrocortisone (Hcrt) (f.c. 1µM), prednisolone (Pred) (f.c. 1µM), fluocinolone acetonide (FA) (f.c. 1µM), and a GR antagonist RU486 (RU) (f.c. 2µM), which were all purchased from Sigma-Aldrich. All reagents above were dissolved in ethanol, and in all experiments, the total solvent concentration was kept similar in all conditions. Recombinant murine tumor necrosis factor (TNF)α was produced in E.coli and purified as described by Vanden Berghe, et al [355]. TNFα was dissolved in medium.

1.2.2. Nuclear-cytoplasmic fractionation

CT5.3hTERT cells were subjected to a 2h treatment with solvent, Dex (1µM) or CpdA (10µM), after which cell lysates were prepared and separated into nuclear and cytoplasmic fractions, as described previously [356]. In short, lysates were treated with hypotonic buffer (20 mM Hepes/KOH pH 7,6; 10 mM NaCl; 20% glycerol; 1.5 mM $MgCl_2$; 0,2 mM EDTA; 0.1% Triton; 25mM β-glycerophosphate; 2mM pefabloc; 10µg/ml aprotinine; 5mM DTT) in order to separate cytoplasmic fraction from the nuclear. Subsequently, the nuclear envelope was ruptured using hypertonic buffer (i.e. hypotonic buffer with addition of NaCl 500mM). Protein concentration was

measured using the Lowry method. A fixed amount of protein from all samples, namely 30 µg, was analyzed via Western Blot.

1.2.3. Reporter gene assay

L929sA cells were stably transfected with the p(IL6κB)350hu.IL6P-luc+ reporter gene using the calcium phosphate precipitation protocol, as described by Vanden Berghe, et al [357]. The cells were induced with solvent, Dex (1µM) or CpdA (1 or 10µM) for 1h and co-treated with TNFα for another 5h. Subsequently, cells were washed with PBS and lysed using TROPIX lysis buffer. Thereafter, we measured the cellular luciferase and galactosidase levels, as described [358,359], at a ParadigmTM Detection Platform (Beckman Coulter®) using SoftMax® Pro 6.1 software. Luciferase activity was corrected for β-galactosidase activity (Galacto- Light kit; Tropix). Results are expressed as relative normalized reporter gene activity in which the condition induced with TNFα was set at 100 and all other conditions were recalculated accordingly.

1.2.4. Cell viability and proliferation assays

To test the cell viability and proliferation of CAFs, cells were seeded and left to adhere for 24h, after which they were treated with solvent, Dex or CpdA, ranging from 0.1µM to 10µM, or left untreated (Ni) for varying time points. To test for potential compound-inducted cytotoxicity, we performed a Lactate Dehydrogenase Activity (LDH) assay (Promega), according to the manufacturer's instructions. To analyze cellular metabolic activity and cell viability we used a classic 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described previously [360]. To assay cell proliferation rate, we performed a sulforhodamine-B (SRB) test, as described [361]. Results were furthermore obtained using a ParadigmTM Detection Platform (Beckman Coulter®) with SoftMax® Pro 6.1 software. Results are expressed as a percentage in which the untreated condition (SRB, MTT) or triton-treated cells (LDH) was set at 100% and all other conditions were recalculated accordingly.

1.2.5. Indirect immunofluorescence

Indirect immunofluorescence microscopy was performed as described previously [268], to visualize GR in CT5.3hTERT cells, after 2h treatment with solvent, Dex (1µM) or CpdA (10µM), using the primary anti-GR (H-300) antibody (Santa Cruz Biotechnology, cat no: sc-8992) together with an Alexa Fluor 488 goat anti-rabbit secondary antibody (Invitrogen Molecular Probes, cat no: A11008). In order to visualize the nuclei we used DAPI (0.4 µg/ml) and UV illumination. Observations were performed using an Axiovert 200M (Zeiss) fluorescence microscope and the images were processed via Axiovision 4.8 Software. Subcellular distribution of GR signal density in cells from various images from two independent experiments was analyzed using ImageJ software

[362]. The value for the solvent condition was set as 1 and all other conditions were recalculated correspondingly to allow ratio comparisons.

1.2.6. Protein lysates and Western blot analysis

For protein analysis, CT5.3hTERT CAFs were induced as indicated in the figure legends. Samples consisted of either collected cells or supernatants from cultured cells. Collected cells were washed twice with PBS. Next, protein lysate samples were prepared using TOTEX buffer (20mM Hepes/KOH pH 7.9; 0.35M NaCl; 20% glycerol; 1% NP40; 1mM $MgCl_2$; 0.5mM EDTA; 0.1mM EGTA; 2mM pefabloc; 10µg/ml aprotinin). After measuring the respective protein concentrations via the Lowry method, 25µg of total protein was denatured, loaded on a SDS-PAGE gel and subjected to the standard Western Blot protocol as described by Santa Cruz (Santa Cruz, CA, USA). Alternatively, SDS sample buffer (50mM Tris pH6.8; 2% SDS; 10% glycerol; bromophenol blue, 100mM DTT) was used to prepare lysates from 10 fold concentrated cell supernatants and a 20µg sample was loaded on a SDS-PAGE gel and subjected to the standard Western Blot protocol.

As primary antibodies, we used anti-GR (H-300) (Santa Cruz Biotechnology, cat no: sc-8992), anti-N-cadherin (BD Biosciences, cat no: 610920), anti-αSMA (Sigma, cat no: A2547), anti-vimentin (Sigma, cat no: V6389), anti-tenascin C (clone BC-24, Sigma, cat no: T2551), anti-TGFβ (R&D Systems, cat no: MAB1835), anti-tubulin (Sigma, cat no: T5168), anti-PARP (BD-Biosciences, cat no: 556494) and anti-GRB2 (C-23, Santa Cruz, cat no: sc-255). To visualize our results we used species-specific HRP-linked secondary antibodies (GE-Healthcare, cat no: NA931V, NA934V), ECL solution (Thermo Scientific) and X-Ray films (GE-Healthcare). To quantify the bands obtained via Western blot analysis, we applied band densitometric analysis via ImageJ software [362]. The area under curve (AUC) of the specific signal was corrected for the AUC of the loading control. The value for the non-induced condition was set as 1 and all other conditions were recalculated correspondingly to allow ratio comparisons.

1.2.7. RT-qPCR

CT5.3hTERT CAFs were induced as indicated in the figure legends. Total RNA was isolated from cells using TRIzol reagent (Life Technologies), according to the manufacturer's instructions. Reverse transcription (RT) was performed using an iScript kit (Bio-Rad), and the obtained cDNA served as a substrate for a quantitative PCR (qPCR) using Lightcycler 480 SYBRGreen I Master reagents (Roche Diagnostics), all processed according to the producer's instruction. qPCR reactions were performed using the Lightcycler® 480 system (Roche Diagnostics), with the following protocol: A) initial denaturation 95°C, 5'; B) 40 cycles of denaturation 95°C, 15'', annealing and elongation 60°C, 45". qPCR reactions were performed in triplicate. The list of primer sequences is available in Addendum 5 (Supplementary Table 4). Specific signal was

normalized to the respective geometric mean of 3 housekeeping gene expression levels (GAPDH, PPIB, 36B4). Displayed results are expressed as relative mRNA expression in which the condition induced with solvent was set as 1 or, where applicable, the condition with TNFα was set as 100. In both cases, all other conditions were recalculated correspondingly.

1.2.8. ELISA HGF/SF

CT5.3hTERT CAFs were treated with solvent, Dex, CpdA, RU486, or a combination of RU486 and Dex, as indicated in the figure legend (Figure 8), and after 48h of induction culture supernatants were collected. In order to measure the total amount of HGF/SF released from the cells, the collected medium samples were analyzed using the HGF Human ELISA Kit (RayBio®, Inc., cat no: ELH-HGF-1) according to manufacturer's instructions. Absorbance was quantified on a ParadigmTM Detection Platform (Beckman Coulter®) using SoftMax® Pro 6.1 software. Results are expressed as relative HGF concentration in cell culture supernatant, where the value of the solvent treatment was set as 1 and all other conditions were recalculated correspondingly to allow ratio comparisons.

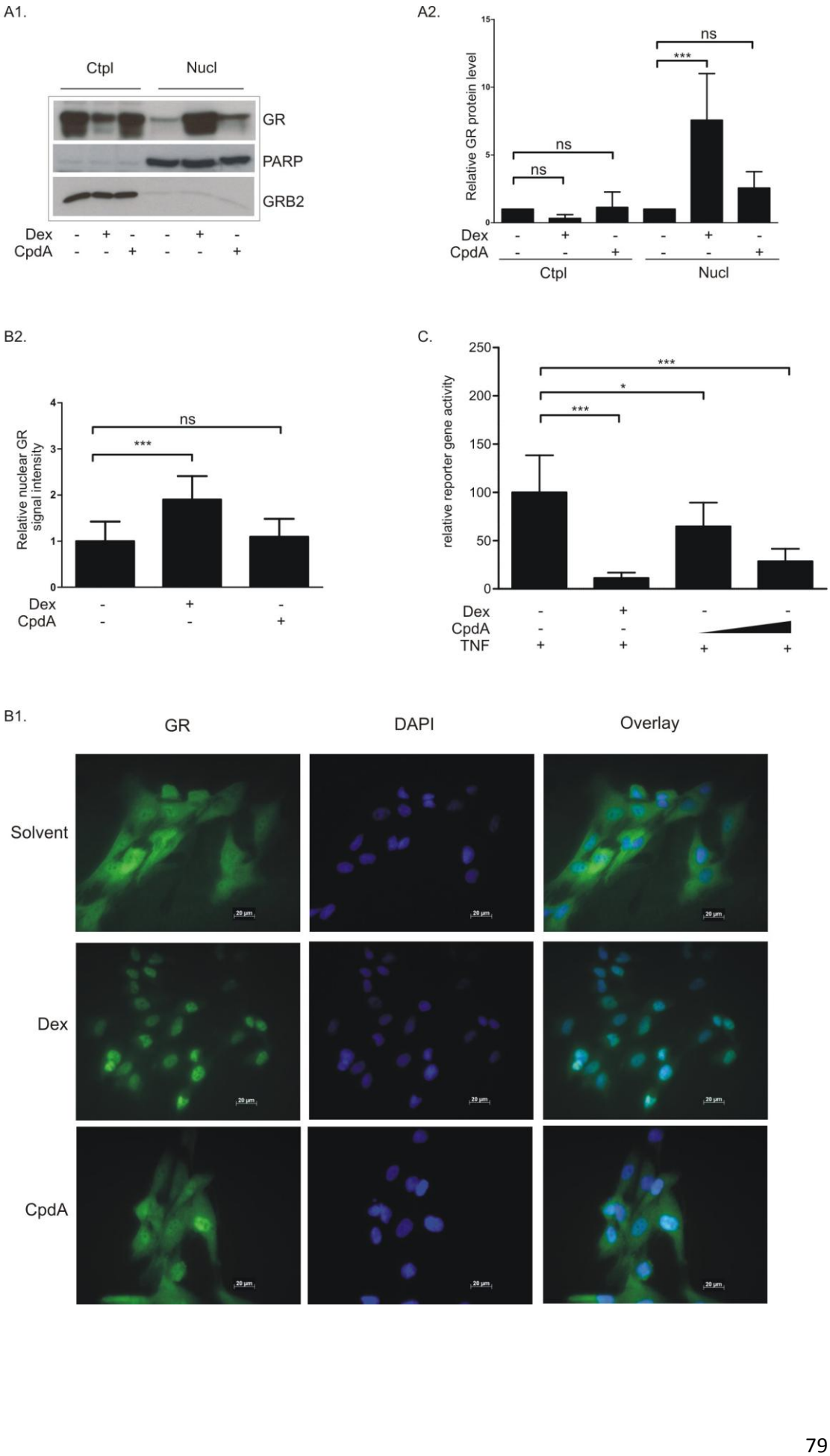
1.2.9. Statistical Analysis

Results are presented ± standard deviations. Statistical analysis was performed using GraphPad Prism 5.03 with a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test, in which a p-value of $p<0.05$ was considered statistically significant, as indicated in the figure legends.

1.3. Results

1.3.1. Dex can translocate GR and repress NFκB-mediated gene expression

To assay the activity of our compounds, we analyzed the ability of the glucocorticoid Dex and the non-steroidal SEGRM CpdA to affect the location of GR and the compounds' ability to inhibit NFκB-mediated gene expression. To that end, we prepared fractionated lysates of CT5.3hTERT cells and visualized GR via Western blot (Figure 17A1). To complement this approach, we used indirect immunofluorescence microscopy, to display the subcellular distribution of GR in CT5.3hTERT cells (Figure 17B1). While Dex can clearly drive GR to the nucleus, CpdA shows to be very weak in affecting GR location in CT5.3hTERT cells (Figure 17A1,A2,B1,B2).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B1.
GR
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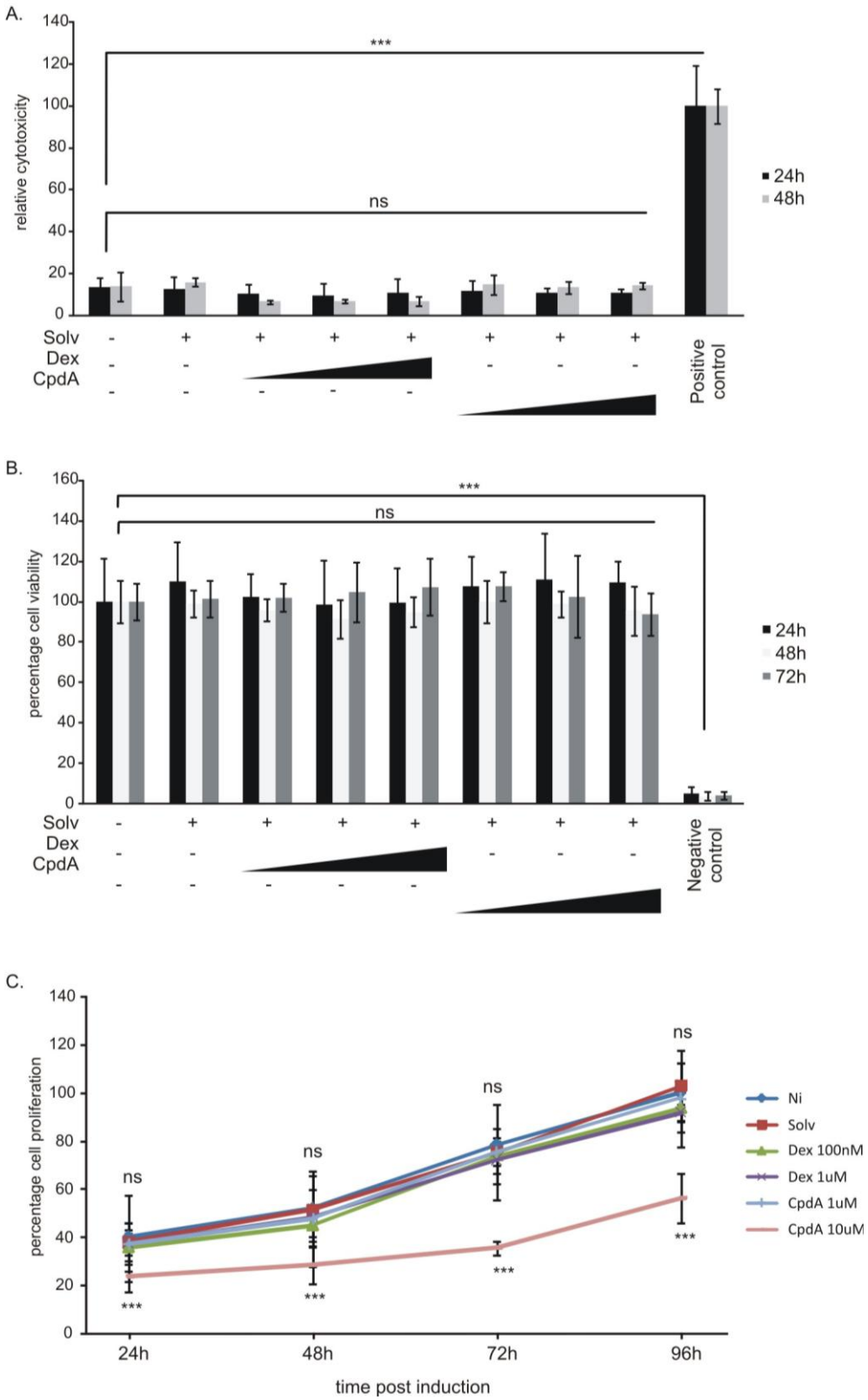
Overlay
Solvent
Dex
CpdA

Figure 17. (previous page) Dex and CpdA can translocate GR and repress NF-κB-mediated gene expression. (A1,A2). CT5.3hTERT cells were treated with solvent, Dex (1μM) or CpdA (10μM) for 2h before cells were lysed and a separation of nuclear and cytoplasmic fractions was performed. Obtained fractions were subjected to Western blot analysis detecting GR, and nuclear PARP and cytoplasmic GRB2 as loading controls. Relative GR intensity, with a correction for sample loading, was determined using band densitometric analysis (ImageJ). The solvent condition was set as 1 in each fraction to allow ratio comparisons. Results are representative of four independent experiments. (B1,B2). CT5.3hTERT cells were seeded on coverslips, starved in Opti-MEM for 24h, and subsequently treated with solvent, Dex (1μM) or CpdA (10μM) for 2h. After washing, fixing, and permeabilizing the cells, indirect immunofluorescence was performed to detect GR. DAPI staining indicates the nuclei. Additionally, we present overlays of both visualizations. This figure is representative of three independent experiments. Relative nuclear GR intensity was determined using densitometric analysis (ImageJ). The solvent condition was set as 1 to allow ratio comparisons. (C). L929sA cells, stably transfected with a p(IL6κB)350hu.IL6P-luc+ gene construct, were treated with solvent, Dex (1μM) or CpdA (1μM or 10μM) for 1h, after which cells were stimulated with TNFα (2000 IU/ml) for another 5h. β-galactosidase control-corrected results are presented as relative reporter gene activity, in which TNF stimulation is set at 100 and all results are recalculated accordingly. Results are the mean ±SD of two independent experiments and statistical analysis on potentially significant differences was performed using a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test. ns not significant, *p<0.05, ***p<0.001.

The Dex and CpdA-mediated effect on GR transrepression activity was confirmed via experiments on L929sA cells, which were stably transfected with a recombinant promoter featuring NFκB response elements (Figure 17C). Stimulation of this promoter with TNFα could be clearly counteracted by Dex and CpdA. In conclusion, despite the impaired ability of CpdA to translocate GR in CT5.3hTERT CAFs, both compounds used are functional and show NFκB-transrepressive properties inL929sA cells, the latter of which corresponds with earlier reports in various cell types [188,256,268,350,352,363-365]. The effects of Dex and CpdA on NFκB-mediated gene expression in CT5.3hTERT CAFs will be investigated more specifically in Figure 21.

1.3.2. Dex and CpdA do not affect CAF viability

To evaluate potential effects of Dex and CpdA on the metabolic activity, viability or cell proliferation rate of CAFs, we performed LDH, MTT and SRB assays upon treatment with different concentrations of Dex and CpdA at different time points (Figure 18). The cytosolic enzyme lactate dehydrogenase (LDH) is released upon loss of membrane integrity and thus cell lysis [366]. Neither Dex nor CpdA show a concentration-dependent or even enhanced cytotoxicity level when CT5.3hTERT CAFs were exposed to these compounds for 24 or 48 hours, indicating that neither Dex nor CpdA compromise the CT5.3hTERT cells' membrane integrity (Figure 18A). As a positive control in this assay, indicating the maximal LDH release potential at a certain time point we used a cell treatment with 20% Triton for 1 hour.


A.
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120
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percentage cell viability
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24h
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time post induction
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Solv
Dex 100nM
Dex 1uM
CpdA 1uM
CpdA 10uM

Figure 18. (previous page) Dex and CpdA do not affect CAF viability. CT5.3hTERT cells were treated with solvent, Dex (0.1µM, 1µM or 10µM) and CpdA (0.1µM, 1µM or 10µM) or left untreated. After the indicated time points cells were subjected to LDH (A), MTT (B) or SRB (C) analyses. (A). Relative cytotoxicity (%) was assayed using an LDH assay. Obtained values were normalized to a positive control of 20% triton-treated cells, which indicates a maximal cytotoxicity. (B). Percentage cell viability was assessed using an MTT assay. Obtained values were normalized to the values obtained using a negative control of non-treated cells, which indicates their maximal viability at a given time point. (C). Percentage cell proliferation was tested using an SRB assay. Obtained values were normalized to a control of non-treated cells at 96h, which indicates their maximal proliferation. Results (ABC) are the mean ±SD of three independent experiments and statistical analysis on potentially significant differences was performed using a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test. ns not significant, ***p<0.001.

In the colorimetric MTT assay, NAD(P)H-dependent cellular oxidoreductase enzymes are used to reflect the metabolic activity of analyzed cells. In viable healthy cells, the yellow tetrazolium salt (MTT) is reduced by these enzymes to a purple soluble formazan [367]. In our analyses, neither Dex nor CpdA significantly affect cellular metabolic activity at 24, 48 and 72 hours post induction (Figure 18B). As expected, our negative control, cells treated with 20% Triton for 1 hour, indeed imposed a steep decrease in cellular metabolic activity, indicating cell death (Figure 18B).

The colorimetric SRB assay determines cell density, based on the cellular protein content. Upon analyzing the cell proliferation rate via this SRB assay, we could show that CpdA (10µM) can slow down CT5.3hTERT cell growth, which results in a significant difference to solvent already after 24hours (Figure 18C). Nevertheless, CpdA (1µM) or Dex at either 100nM or 1µM does not profoundly affect the proliferation rate of CT5.3hTERT cells (Figure 18C).

In conclusion, neither Dex nor CpdA significantly affect CT5.3hTERT CAF viability, but CpdA can negatively impact the growth rate of these cells.

1.3.3. Effect of Dex and CpdA on the expression pattern of myofibroblastic markers

CAFs can differentiate from different cell types, such as fibroblasts, epithelial, endothelial and mesenchymal stem cells. However, as common characteristic these CAFs, regardless of their origin, express αSMA and vimentin markers [76]. In order to test how Dex and CpdA influence these myofibroblastic characteristics, we performed mRNA and protein analysis of αSMA and vimentin (Figure 19A-D). Neither Dex nor CpdA treatment affects expression of αSMA on both transcription and translation levels in CT5.3hTERT CAFs (Figure 19A,B,D). Vimentin mRNA expression is, however, slightly affected by various glucocorticoids: dexamethasone (Dex), hydrocortisone (Hcrt), prednisolone (Pred), fluocinolone acetonide (FA), and a CpdA treatment (Figure 19C). This effect is reversed by a steroidal GR antagonist RU486 (RU) and it does not persist on protein level (Figure 19D). In conclusion, neither Dex nor CpdA impact CAFs' typical features.

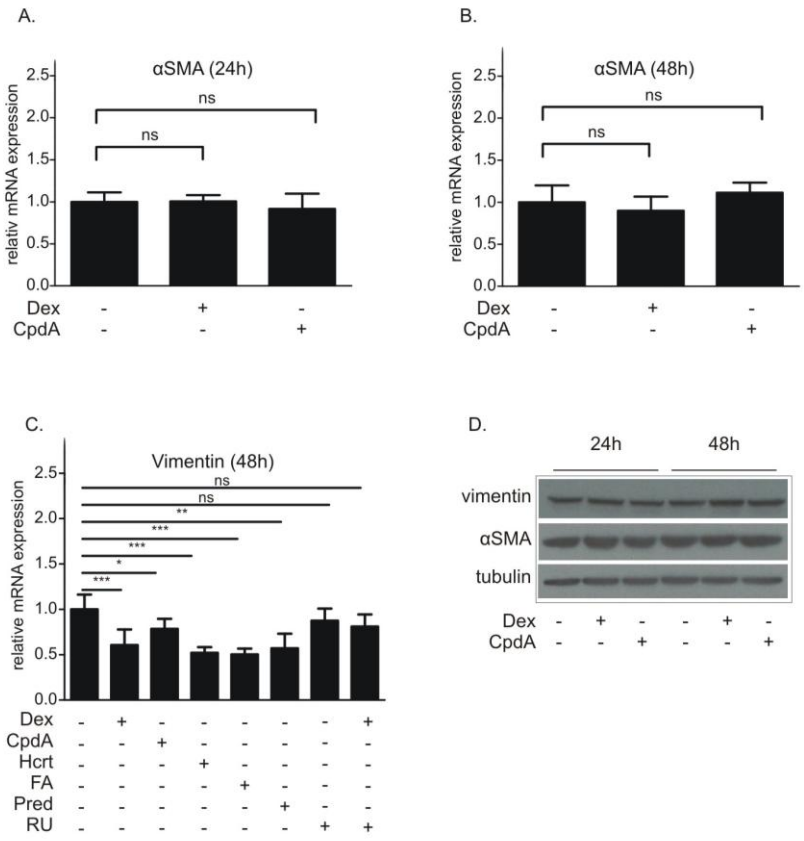


Figure 19. Effect of Dex and CpdA on the expression pattern of myofibroblastic markers. CT5.3hTERT were treated with solvent, Dex (1µM) or CpdA (10µM) for 24h or 48h (A,B) or with solvent, Dex (1µM), CpdA (10µM), Hcrt (1µM), FA (1µM), Pred (1µM), or RU (2µM) or co-treated with Dex (1µM) and RU (2µM) for 48h (C). Isolated mRNA was subjected to RT-qPCR assaying αSMA and vimentin mRNA levels, and results were normalized to the respective geometric mean of GAPDH, PPIB and 36B4 household genes' mRNA levels. The solvent condition was set at 1 and results were recalculated accordingly. Results (A,B,C) are the mean ±SD of three independent experiments and statistical analysis on potentially significant differences was performed using a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test. ns not significant, *p<0.05, **p<0.01, ***p<0.001. (D). CT5.3hTERT cells were treated with solvent, Dex (1µM) or CpdA (10µM) for 24 or 48h. Cell lysates were subjected to Western Blot analysis to detect αSMA, vimentin, and the loading control tubulin. Results are representative of at least three independent experiments.

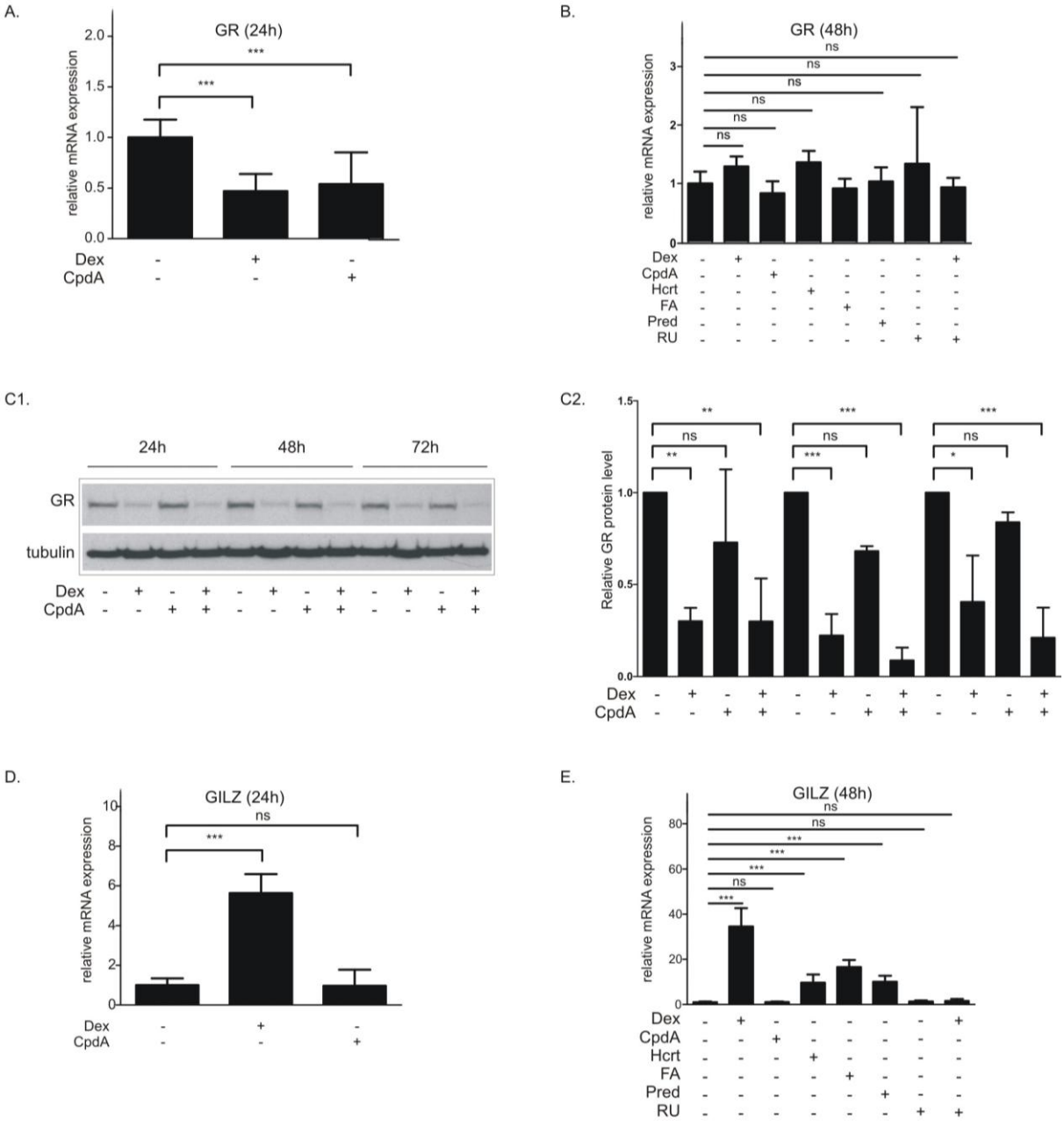


Figure 20. Glucocorticoids trigger GR transactivation and diminish GR expression in CAFs. CT5.3hTERT were treated with solvent, Dex (1µM) or CpdA (10µM) for 24h (A,D) or with solvent, Dex (1µM), CpdA (10µM), Hcrt (1µM), FA (1µM), Pred (1µM), or RU (2µM) or co-treated with Dex (1µM) and RU (2µM) for 48h (B,E). Isolated mRNA was subjected to RT-qPCR assaying GR and GILZ mRNA levels, and results were normalized to the respective geometric mean of GAPDH, PPIB and 36B4 household genes' mRNA levels. The solvent condition was set at 1 and results were recalculated accordingly. Results (A,B,D,E) are the mean ±SD of at least three independent experiments and statistical analysis on potentially significant differences was performed using a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test. ns not significant, ***p<0.001. (C1,C2). CT5.3hTERT cells were treated with solvent, Dex (1µM), CpdA (10µM) or co-treated with Dex (1µM) and CpdA (10µM) for 24, 48 or 72h. Cell lysates were subjected to Western Blot analysis for the detection of GR and the loading control tubulin. Relative GR intensity, with a correction for sample loading, was determined using band densitometric analysis (ImageJ). The solvent condition was set as 1 at each time point to allow ratio comparisons. The displayed results are representative of at least three independent experiments.

1.3.4. Glucocorticoids trigger GR transactivation and diminish GR expression in CAFs

In order to test how the glucocorticoids Dex, Hcrt, Pred, FA, a GR antagonist RU486 (RU) and a SEGRM CpdA influence GR expression and activity in CT5.3hTERT CAFs, we performed mRNA and protein analyses of GR and an mRNA analysis of a typical GR target gene, i.e. glucocorticoid-inducible leucine zipper (GILZ) (Figure 20).

GR mRNA levels are significantly diminished 24 hours post induction by both Dex and CpdA (Figure 20A). However, this initial difference does not continue after 48 hours (Figure 20B). Similar results at 48 hours are obtained with other GCs (Figure 20B).

Long-term treatment with Dex is known to trigger homologous downregulation of GR [368]. Also in the CAF cell line, prolonged treatment with a GC, in this case Dex, results in diminished protein levels of the receptor (Figure 20C1,C2). Although CpdA has a temporary inhibitory effect on GR mRNA levels, treatment of CT5.3hTERT CAFs with CpdA ultimately does not lead to a decrease in GR protein levels. As the GR levels, and GR homologous downregulation can play an important role in GC resistance [195], we explored whether simultaneous exposure of CT5.3hTERT CAFs with Dex and CpdA could prevent GC-mediated GR downregulation. Unfortunately, our results show that CpdA cannot protect GR from a Dex-induced downregulation (Figure 20C1,C2).

Notwithstanding the clear downregulation of GR, the minimal GR levels that remain present show to be sufficient to allow a strong upregulation of GILZ mRNA levels after 24 and even 48 hours of GC treatment (Figure 20D,E). This effect is completely antagonized by a co-treatment with the GR antagonist RU486 (Figure 20E). In line with the nature of CpdA as a selective GR modulator, treatment with CpdA does not influence GILZ levels after 24 or 48 hours induction (Figure 20D,E).

1.3.5. Both Dex and CpdA diminish pro-inflammatory gene expression in CAFs

GR's anti-inflammatory properties are mostly mediated via interference of GR with another transcription factor, such as NFκB or AP-1, which leads to suppression of multiple pro-inflammatory genes [188]. In our study we tested whether in CT5.3hTERT CAFs the activated GR holds anti-inflammatory functions upon stimulation with TNFα. In our analyses multiple pro-inflammatory genes are strongly up-regulated by TNFα, while this effect is counteracted by GCs for all cytokines and chemokines tested, i.e. RANTES, ICAM, MCP-1, IL-1β and TNFα, except for IL-6 (Figure 21A-F). For the latter cytokine, we even recorded that CpdA could enhance its expression level (Figure 21A). Both Dex and CpdA decrease the expression of the chemokine RANTES and the adhesion molecule ICAM (Figure 21D,F). However, in contrast with previous research in adenocarcinomic human alveolar epithelial cells (A549) and in rheumatoid arthritis synovial fibroblasts (FLS) [270,365], CpdA is unexpectedly unable to significantly repress gene expression levels of MCP-1, IL-1β and TNFα (Figure 21B,C,E), indicating cell-specific mechanisms.

When analyzing the natural inhibitors of TNFα-stimulated NFκB activation, we observe that the GC Dex has a trend to stimulate the gene expression of IκBα (NFκBIa) and A20 (TNFAIP3) after 6h of induction (Figure 21G-H). Given a multitude of earlier likewise, but cell-specific observations for IκBα [245,369], we also assayed later time points and could confirm that this trend after 24h and 48h of induction accumulates into a statistically significant difference in solvent- vs. Dex-stimulated cells for both IκBα and A20 levels (Addendum 1, Supplementary Figure 1). Within the line of expectations, given the presence of both a GR- and an NFκB-responsive binding motif in the promoter of the IκBα gene and earlier corresponding results [365,369,370], combined exposure of CT5.3hTERT CAFs to an NFκB-activating TNFα stimulus and Dex, does not enhance mRNA levels of IκBα further. However, CpdA, unlike earlier reports in A549 cells [365], does not inhibit the TNFα-stimulated IκBα and A20 gene expression (Figure 21G-H), again suggesting a cell-specific set of events in CT5.3hTERT cells.

1.3.6. Dex negatively influences the expression of several factors involved in cancer progression

To research whether GCs and/or SEGRMs could potentially influence key molecules which are secreted by CAFs, we investigated several factors known to play a role in tumor progression. TGFβ which is an autocrine or paracrine growth factor, indirectly also acts as a strong pro-invasive factor for cancer cells, as it stimulates transdifferentiation of fibroblasts into myofibroblasts, which in turn, promote cancer invasion [76]. TNC and HGF/SF are proteins secreted by CAFs, which are known to provide pro-invasive signals to cancer cells [77]. N-cad, a transmembrane protein upregulated by TGFβ, plays a critical role in CAF invasion and migration, and therefore indirectly in tumor progression [131].

The CT5.3hTERT cell levels of N-cadherin mRNA were clearly decreased by Dex, but not by CpdA (Figure 22A). However, upon analyzing the corresponding protein levels we could not detect any Dex- or CpdA-mediated change in N-cadherin protein levels at 24, 48 or 72 hours of induction (Figure 22B).

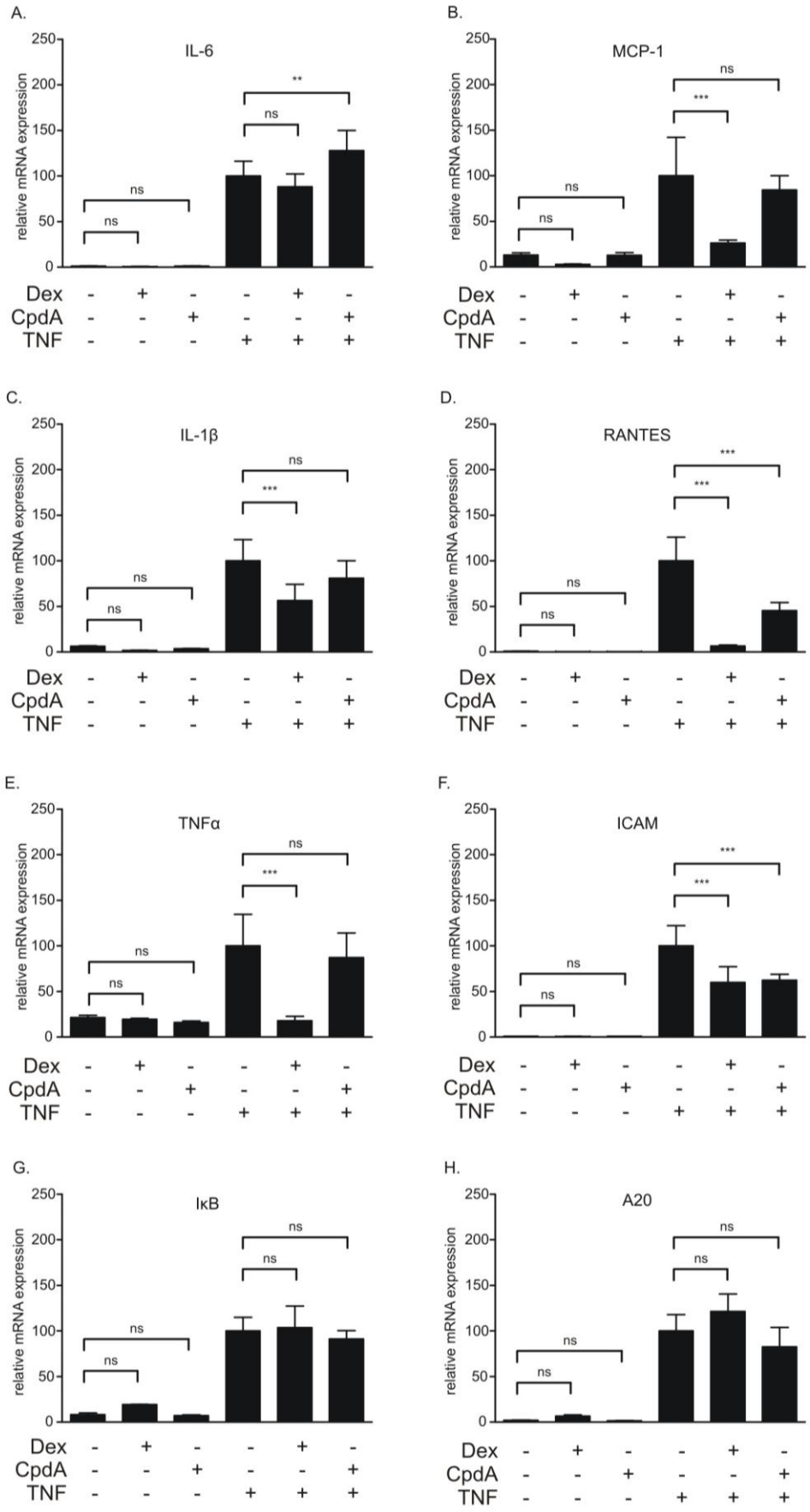
Our analyses further show that TGFβ mRNA expression in CT5.3hTERT cells is only slightly affected by Dex after 24 and 48 hours of induction, but not 6 hours of induction (Figure 23A-C). Treatment of these cells with CpdA at any time point leaves TGFβ expression levels unchanged (Figure 23A-C). Upon analyzing the protein levels of secreted TGFβ, we detected that Dex could negatively impact both the TGFβ precursor and activated TGFβ protein levels (Figure 23D). As TGFβ stimulates N-cad expression in CAFs [131], the decreased levels of secreted active TGFβ protein may explain downregulation of N-cad mRNA levels (Figure 22A).

HGF/SF (Figure 24) and TNC (Figure 25) behave quite similarly. Both factors' mRNA levels are strongly downregulated when exposed to Dex, but not with a CpdA treatment. For Dex, this

negative impact persists on the protein levels of HGF and TNC, which can be counteracted by the GR antagonist RU486, albeit incompletely (Figure 24D-25D). Of note, also the inhibitory effect of Dex on the gene expression levels of TNC can be significantly antagonized by RU486 (Addendum 1, Supplementary Figure 2). However, the protein concentrations of HGF and TNC can be moderately decreased by CpdA (Figure 24D, 25D).

In conclusion, notwithstanding Dex can inhibit the mRNA levels of TGFβ, N-cad, TNC and HGF to varying extents, CpdA could never inhibit the expression levels of all of these genes (Figure 22-25), suggesting the involvement of indirectly acting GR transactivation mechanisms.

Figure 21. (next page) Both Dex and CpdA diminish pro-inflammatory gene expression in CAFs. CT5.3hTERT were treated with solvent, Dex (1μM), CpdA (10μM) for 1h either or not co-treated with TNFα (2000 IU/ml) for another 5h. Total mRNA was subjected to RT-qPCR assaying IL-6 (A), MCP-1 (B), IL-1β (C), RANTES (D), TNFα (E), ICAM (F), IκBα (G) and A20 (H) mRNA levels, and results were normalized to the respective geometric mean of GAPDH, PPIB and 36B4 household genes' mRNA levels. The condition induced with solely TNFα was set at 100 and results were recalculated accordingly. Results are the mean ±SD of three independent experiments and statistical analysis on potentially significant differences was performed using a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test. ns not significant, **p<0.01***p<0.001.


A.
IL-6
B.
MCP-1
C.
IL-1β
D.
RANTES
E.
TNFα
F.
ICAM
G.
IκB
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A20
relative mRNA expression
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50
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150
200
250
ns
**
***
Dex
CpdA
TNF

1.4. Discussion

In this study we investigated the impact of a classic glucocorticoid (GC) dexamethasone (Dex) and a selective glucocorticoid receptor modulator (SEGRM) compound A (CpdA) on the biology of colon cancer-derived CAFs. The stromal environment of the tumor plays an essential role in cancer cell proliferation, invasion and metastasis. Via paracrine secretion of multiple factors, among which TGFβ, cancer cells recruit myofibroblasts from surrounding precursors, which in turn are able to modulate the epithelial-to-mesenchymal transition (EMT) of cancer cells, ultimately facilitating invasion [76,347]. The glucocorticoid receptor (GR) is responsible for anti-inflammatory actions via transactivation or transrepression mechanisms, of which the latter mechanisms results in downregulation of many pro-inflammatory genes including cytokines, enzymes and adhesion molecules [188,245,349]. Many of those factors are involved in the cross-talk between cancer cells and their stromal environment [76].

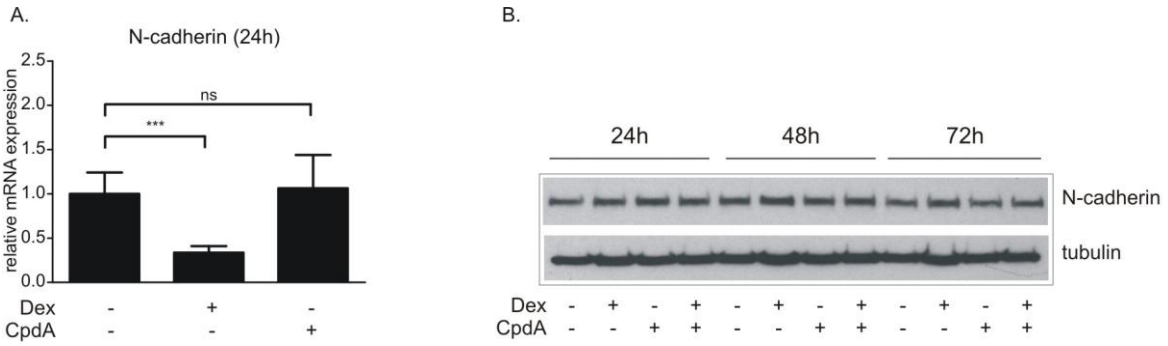

Figure 22. Dex decreases the N-cadherin mRNA levels, but leaves its protein unaffected. (A). CT5.3hTERT cells were treated with solvent, Dex (1μM) or CpdA (10μM) for 24h. Total mRNA was subjected to RT-qPCR assaying N-cadherin mRNA levels, and results were normalized to the respective geometric mean of GAPDH, PPIB and 36B4 household genes mRNA levels. The solvent condition was set at 1 and results were recalculated accordingly. Results are the mean ±SD of three independent experiments and statistical analysis on potentially significant differences was performed using a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test. ns not significant, ***p<0.001. (B). CT5.3hTERT cells were treated with solvent, Dex (1μM), CpdA (10μM) or co-treated with Dex (1μM) and CpdA (10μM) for 24, 48 and 72h. Cell lysates were subjected to Western Blot analysis for detection of N-cadherin and the loading control tubulin. Results are representative of three independent experiments.

Our study shows that both Dex and CpdA do not affect CAF viability, although a high concentration of CpdA can impair its cell growth rate (Figure 18). Both compounds activate GR, which is reflected by their ability to translocate the GR, although in CpdA's case this effect is very modest (Figure 17A). Earlier, CpdA was reported to translocate GR into the nucleus, although with a slightly (in A549 cells) or clearly (in LNCaP-GR cells and fibroblast-like synoviocytes) lower efficacy than a classic GC [268,269,353]. Both Dex and CpdA can impose their anti-inflammatory properties at specific target genes (Figure 21). CpdA can act as a selective GR modulator in CAFs, as combined with its repressive action on NFκB-mediated gene expression (Figure 17C, 21), it fails

to stimulate GR transactivation, characteristic for steroidal GCs (Figure 20D,E). Although both compounds are functional in CAFs, only a select number of pro-inflammatory genes are affected by repressive actions of activated GR (Figure 21). Dex and CpdA downregulate TNFα-stimulated expression of RANTES and ICAM, only Dex represses MCP-1, IL-1β and TNFα, and none of our GR-modulating compounds downregulate IL-6 (Figure 21). This phenomenon might be caused by the fact that the anti-inflammatory actions of GR are composed of a layered multitude of mechanisms depending on the transcription factor that is involved and the cell-specific background in which they are recorded [245,365].

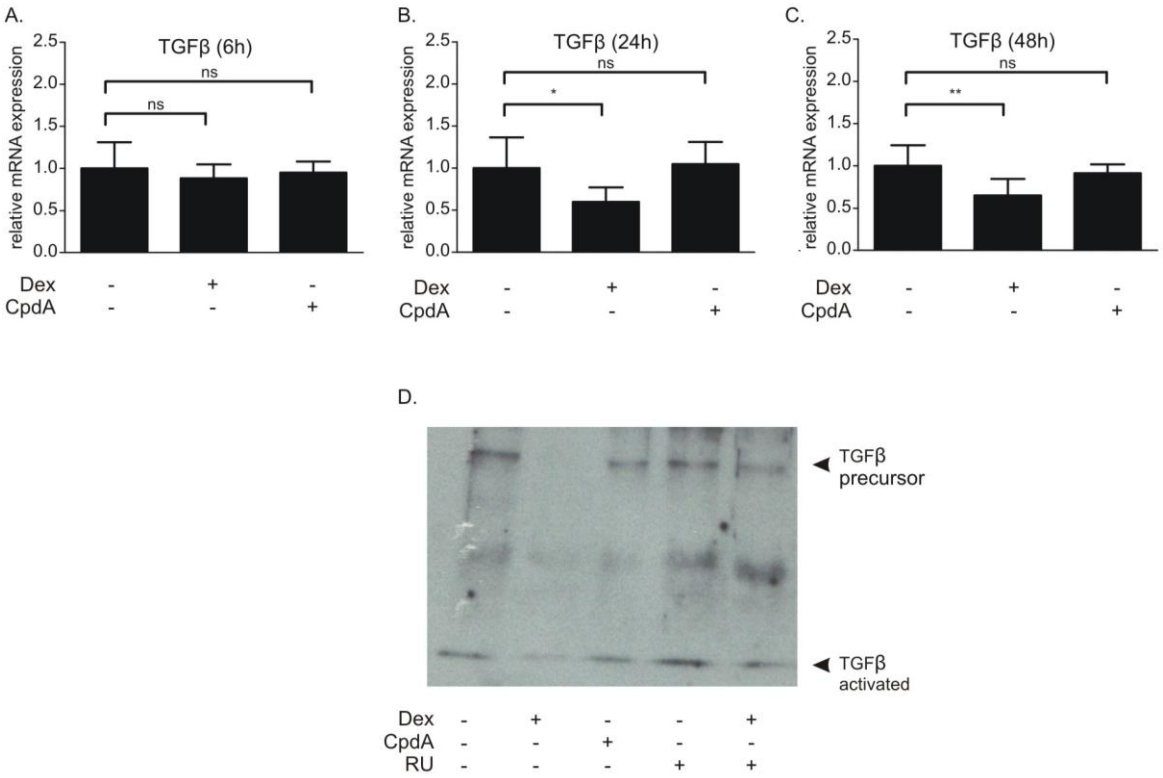

Figure 23. Dex decreases TGFβ levels. (A,B,C). CT5.3hTERT cells were treated with solvent, Dex (1μM) or CpdA (10μM) for 6 (A), 24 (B) or 48h (C). Total mRNA was subjected to RT-qPCR assaying TGFβ mRNA levels, and results were normalized to the respective geometric mean of GAPDH, PPIB and 36B4 household genes' mRNA levels. The solvent condition was set at 1 and results were recalculated accordingly. Results are the mean ±SD of three independent experiments and statistical analysis on potentially significant differences was performed using a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test. ns not significant, *p<0.05, **p<0.01. (D). CT5.3hTERT cells were treated with solvent, Dex (1μM), CpdA (10μM), RU (2μM) or co-treated with Dex (1μM) and RU (2μM). After 48h cell culture supernatant was collected and concentrated 10 fold. The protein fractions of these concentrated supernatants were subjected to Western Blot analysis for detection of TGFβ. Results are representative of three independent experiments.

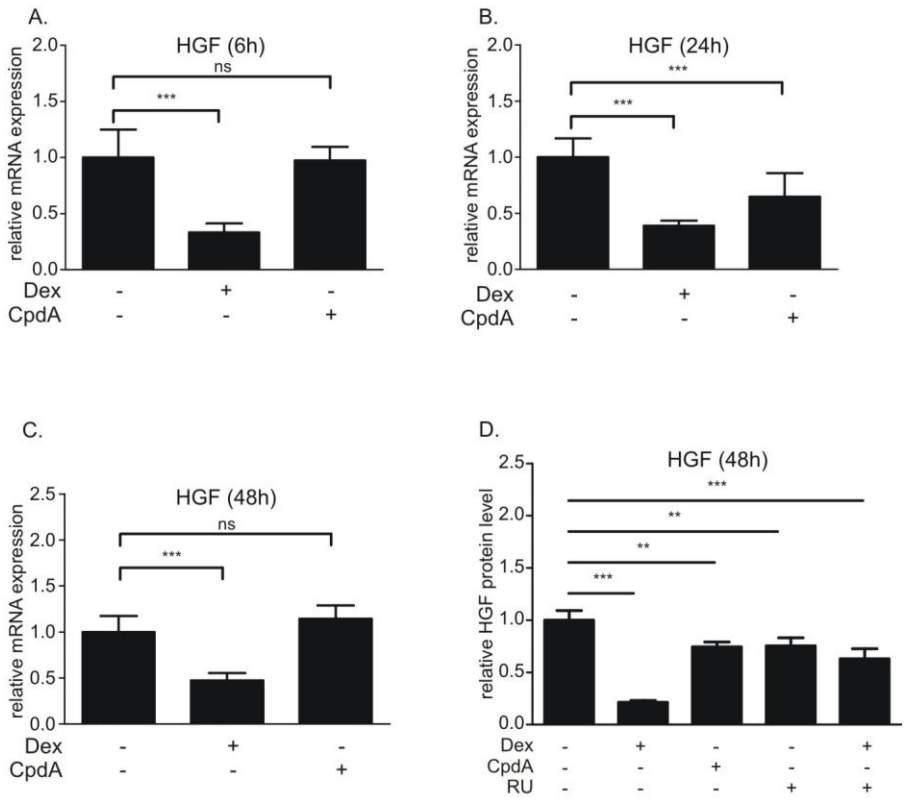

Figure 24. Dex reduces the expression of hepatocyte growth factor. CT5.3hTERT were treated with solvent Dex (1µM) or CpdA (10µM) for 6h (A), 24h (B) or 48h (C). Total mRNA was subjected to RT-qPCR assaying HGF mRNA levels, and results were normalized to the respective geometric mean of GAPDH, PPIB and 36B4 household genes' mRNA levels. The solvent condition was set at 1 and results were recalculated accordingly. Results are the mean ±SD of three independent experiments and statistical analysis on potentially significant differences was performed using a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test. ns not significant, ***p<0.001. (D). CT5.3hTERT cells were treated with solvent, Dex (1µM), CpdA (10µM), or RU (2µM) or co-treated with Dex (1µM) and RU (2µM). After 48h cell culture supernatant was collected and analysed via an HGF ELISA. The solvent condition was set at 1 and results were recalculated accordingly. Results are the mean ±SD of two independent experiments and statistical analysis on potentially significant differences was performed using a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test. **p<0.01, ***p<0.001.

Sustained exposure to glucocorticoids causes GR homologous downregulation, which in turn plays an essential role in causing glucocorticoid resistance [195,368,371]. Our results show that in CAFs the long-term exposure to GCs leads to homologous downregulation of GR, which does not occur with CpdA treatment (Figure 20), as was also shown before in fibroblast-like synoviocytes [352]. However, we now also established that simultaneous treatment of CAFs with CpdA and the GC Dex does not prevent this downregulation phenomenon. Given the fact that CpdA can also decrease the GR mRNA level, further research into the precise cause of the absence of a CpdA-induced GR downregulation remains required.

Epithelial-to-mesenchymal transition (EMT) is a process in which epithelial cells lose their typical morphology and gain the ability to become motile and invasive. EMT is characteristic for embryo development and wound healing, but it also plays a significant role in neoplastic progression of

human epithelial tumors and in fibrosis [121,372]. Changes associated with EMT include downregulation of typical epithelial markers (e.g. cytokeratin, E-cadherin), which results in loss of cell polarity, and the acquisition of typical mesenchymal markers (e.g. expression of N-cad, vimentin, fibronectin) [121,373].

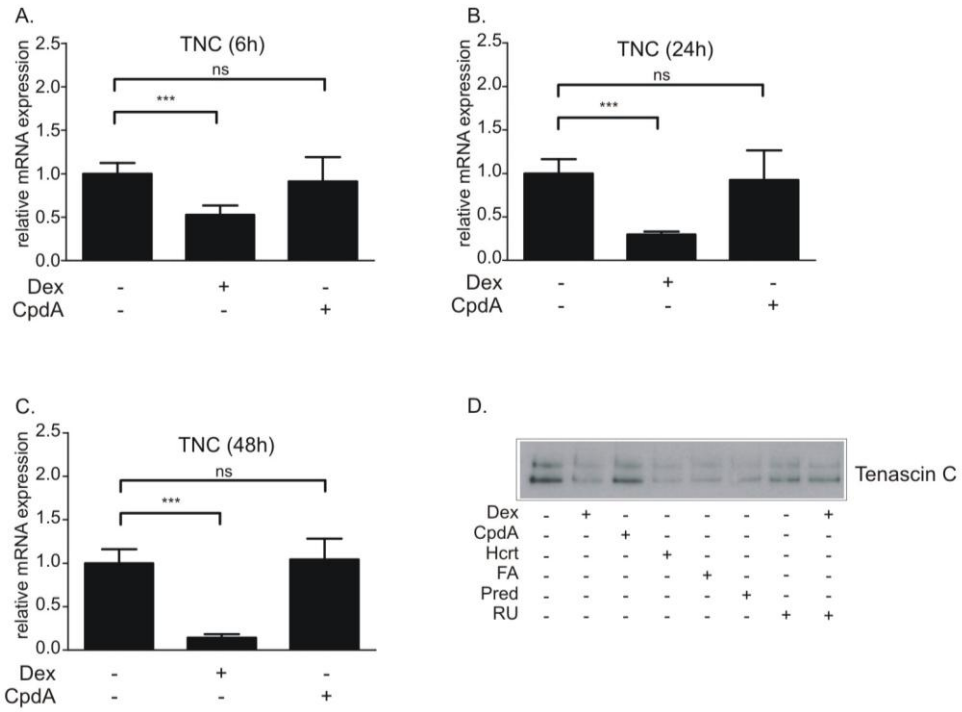

Figure 25. Dex decreases the expression of tenascin C. (A,B,C). CT5.3hTERT were treated with solvent, Dex (1µM) or CpdA (10µM) for 6 (A), 24 (B) or 48 (C) h. Total mRNA was subjected to RT-qPCR assaying TNC mRNA levels, and results were normalized to the respective geometric mean of GAPDH, PPIB and 36B4 household genes' mRNA levels. The solvent condition was set at 1 and results were recalculated accordingly. Results are the mean ±SD of three independent experiments and statistical analysis on potentially significant differences was performed using a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test. ns not significant, ***p<0.001. (D). CT5.3hTERT cells were treated with solvent, Dex (1µM), CpdA (10µM), Hcrt (1µM), FA (1µM), Pred (1µM), RU (2µM) or co-treated with Dex (1µM) and RU (2µM). After 48h cell culture supernatant was collected and concentrated 10 fold. The protein fractions of these concentrated supernatants were subjected to Western Blot analysis for detection of TNC. Results are representative of three independent experiments.

Since CAFs share characteristics with smooth-muscle cells and fibroblasts, they express vimentin, αSMA and a mesenchymal marker N-cad. The latter path-finding molecule is involved in migration and invasion, and in CAFs it contacts with the αSMA cytoskeleton [35,131]. Both CAFs and invasive cancer cells express N-cadherin, which enables them to sense the surrounding environment and to promote invasion into extracellular matrix [374]. Moreover, N-cad and αSMA are upregulated in CAFs by TGFβ [131]. None of these typical myofibroblastic proteins, i.e. vimentin, αSMA or N-cad are affected by Dex or CpdA treatment (Figure 20, 22). Downregulation of mRNA with constant protein levels of N-cad might be caused by less effective metalloproteinase-dependent cleavage of N-cad [375].

As EMT is a complex net of cellular and molecular mechanisms, there are multiple pathways involved in its regulation, mediated by factors among which TGFβ and NFκB play a role. Myofibroblasts recruited by cancer cells are able to trigger EMT in epithelial cancers via the secretion of multiple cytokines, chemokines and molecules involved in remodeling the extracellular matrix [76,347]. Many of those factors, such as RANTES and IL-6, are up-regulated via NFκB signaling during the process of inflammation. An activated GR acts as a suppressor for NFκB actions via transrepressive mechanisms, which makes it the first line of defense against inflammation [370]. Therefore, repressing the expression of pro-inflammatory factors is important to control the cross-talk between CAFs and cancer cells at the invasion front.

HGF is a mesenchyme-derived factor with morphogenic and mitogenic activities on endothelial and epithelial cells [376]. HGF is secreted by CAFs and is able to activate c-Met receptor tyrosine kinase of a target cell, which in turn leads to invasive growth [377]. Our results present that due to GC treatment both mRNA and protein levels of secreted HGF in cell culture supernatant are strongly diminished (Figure 24, Addendum 1, Supplementary Figure 2). It was reported before that GCs also downregulate expression of HGF in human lung fibroblasts and leukemic cells [378], and in human osteoblast-like cells [379], but this was never researched in CAFs.

TNC is an extracellular matrix glycoprotein commonly expressed in embryonic and adult tissues which undergo active remodeling. During development, disease or injury or in case of cancer, TNC is often expressed by myofibroblasts and with a low-affinity it can bind to and activate epidermal grow factor receptor (EGFR) [77,380,381]. Our results show a strong decrease in TNC mRNA and protein levels after treatment with Dex (Figure 25). CpdA, however, has no impact on TNC mRNA, but it slightly affects protein levels. The Dex-mediated decrease of TNC is only partially reversed by co-treatment with RU486 (Figure 25, Addendum 1, Supplementary Figure 2). Moreover, a treatment with RU486 alone also leads to down-regulation of TNC. This phenomenon might be explained by the fact that RU486 acts as a strong antagonist for transactivation actions of GR, but it has a weaker effect on GR transrepression and it also carries partial agonist properties [238].

TGFβ is a cytokine that controls many cellular mechanisms such as proliferation and cell differentiation. Depending on the cell state, it can both lead to tumor suppression or promotion. In normal epithelial cells it serves as a growth inhibitor, however, in absence of the TGFβ receptor, which is frequently inactivated in human colon carcinomas due to mutations, production of this factor promotes recruitment of surrounding stromal cells, without affecting cancer cells themselves [8,100]. Together with the platelet-derived growth factor (PDGF) TGFβ is responsible for triggering wound-healing and appearance of myofibroblasts [382]. Recruited myofibroblasts, among many other factors also secrete TGFβ, which in turn stimulates directly or indirectly other surrounding cells, as well as processes of angiogenesis and escape of the

immunosurveillance [100,383]. Our results show that in CAF TGFβ mRNA expression is only slightly affected by Dex treatment, and not by CpdA (Figure 23A-C, Addendum 1, Supplementary Figure 2). Protein analysis, however, shows that levels of secreted TGFβ precursor and active TGFβ molecule strongly decrease upon Dex treatment, but not CpdA (Figure 23D).

Glucocorticoids are widely used in inflammatory afflictions, but also in anti-cancer therapy [204,348]. However, long-term use of GCs might carry deleterious effects on patients' health, can cause GC resistance [371] and accelerate cancer progression [278,280,354]. Hence, it is important to continue to investigate the molecular mechanisms of GCs in tumors, but also in their environment. In this study we have investigated the role of GR modulation in cancer-surrounding cells, namely colon cancer-derived CAFs, which are known to strongly influence cancer cells via cell-to-cell or paracrine signaling [76]. In these CAFs, GC treatment results in a significant downregulation of several factors involved in cancer invasion and progression, i.e. TGFβ, TNC and HGF/SF. Notwithstanding the inhibitory effects on the expression of these factors via GC-bound GR, the selective GR modulator CpdA has only a very modest impact on the gene and protein levels of these cancer-promoting factors, which is potentially caused by either the involvement of a specific GC/GR or GR transactivation component or caused by the observed impaired CpdA-driven GR translocation in these CAFs. However, CpdA does clearly slow down CAF cell growth. Although current research on selective GR modulators already creates the opportunity to dissociate transactivating actions of GR from the transrepressive ones, further studies are still needed to fully explore this mechanism and the limitations of these compounds.

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CHAPTER 2: ARTICLE 2.

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Colon cancer-derived myofibroblasts increase endothelial cell migration by glucocorticoid-sensitive secretion of a pro-migratory factor

Zuzanna Drebert [a,b], Mark MacAskill [c], Dahlia Doughty-Shenton [d], Karolien De Bosscher [b,e], Marc Bracke [a,b], Patrick W.F. Hadoke [c,*,1], Ilse M. Beck [a,b,1,2]

[a] Laboratory of Experimental Cancer Research, Department of Radiation Oncology & Experimental Cancer Research, Ghent University, Ghent, Belgium
[b] Cancer Research Institute Ghent (CRIG), Ghent, Belgium
[c] University/BHF Centre for Cardiovascular Science, The Queen's Medical Research Institute, University of Edinburgh, Edinburgh, United Kingdom
[d] Edinburgh Phenotypic Assay Centre, The Queen's Medical Research Institute, University of Edinburgh, Edinburgh, United Kingdom
[e] Receptor Research Laboratories, Nuclear Receptor Lab (NRL), VIB Department of Medical Protein Research, Ghent University, Ghent, Belgium

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Abstract

Angiogenesis is important in cancer progression and can be influenced by stromal myofibroblasts, also known as cancer-associated fibroblasts (CAFs). We addressed the hypothesis that glucocorticoids indirectly affect angiogenesis by altering the release of pro-angiogenic factors from colon cancer-derived CAFs.

Our study shows that glucocorticoids reduced prostanoids, urokinase-type plasminogen activator (uPA) and angiopoietin-like protein-2 (ANGPTL2) levels, but increased angiogenin (ANG) in supernatant from human CT5.3hTERT colon cancer-derived CAFs. Conditioned medium from solvent- (CM^{CTRL}) and dexamethasone (Dex)-treated (CM^{DEX}) CAFs increased human umbilical vein endothelial cell (HUVEC) proliferation, but did not affect expression of pro-angiogenic factors or tube-like structure formation (by HUVECs or human aortic ECs). In a HUVEC scratch assay CM^{CTRL}-induced acceleration of wound healing was blunted by CM^{DEX} treatment. Moreover, CM^{CTRL}-induced neovessel growth in mouse aortic rings *ex vivo* was also blunted using CM^{DEX}. The latter effect could be ascribed to both Dex-driven reduction of secreted factors and potential residual Dex present in CM^{DEX} (indicated using a dexamethasone-spiked CM^{CTRL} control). A similar control in the scratch assay, however, revealed that altered levels of factors in the CM^{DEX}, and not potential residual Dex, were responsible for decreased wound closure.

In conclusion, our results suggest that glucocorticoids indirectly alter endothelial cell function during tumor development *in vivo*.

2.1. Introduction

Angiogenesis, the formation of new blood vessels from an existing vascular network [85], is essential for embryonic growth. In healthy adults angiogenesis is restricted to discrete physiological processes (e.g. the regulation of the reproductive tract, muscle growth) and contributes to wound healing [44]. Excessive or impaired angiogenesis has also been implicated in disease pathogenesis (e.g. in malignant or inflammatory disorders [44]), and is associated with promotion of tumor growth and metastasis. Consequently, the potential of angiogenesis as a therapeutic target (e.g. in cancer [44,85,96], retinopathy [334] and tissue ischemia [384]) has attracted considerable research interest.

Tumors use blood vessels not only as a source of nutrients and oxygen, but also to transport cancer cells to establish a new, metastatic site [43]. Cancer cells can directly modulate angiogenesis via secretion of pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), angiopoietins, basic fibroblast growth factor (bFGF), interleukins (ILs) or transforming growth factors (TGFs) [86,96]. Epithelial tumors consist of cancer cells and a surrounding microenvironment composed of an extracellular matrix, stromal cells, inflammatory cells and endothelial cells (ECs). All these components play an important role during tumor development [138]. Myofibroblasts recruited by cancer cells, also known as cancer-associated fibroblasts (CAFs) are present at the invasive edge of the tumor and share properties of both smooth-muscle cells and fibroblasts. Myofibroblasts, which are essential during wound healing and embryonic development [346], can also influence tumor progression [76,140] either directly, through paracrine signaling to cancer cells, or indirectly, by modulation of protease activity, modulation of extracellular matrix remodeling, and recruitment of immune cells [76,138]. CAFs also have the potential to alter EC function and influence tumor angiogenesis [138,140]. In breast cancer, CAFs promote vascularization by recruiting endothelial progenitor cells to the tumor via increased release of stromal-cell derived factor 1 (SDF)-1 [139]. Moreover, prostaglandin (PG)E_2-stimulated intestinal sub-epithelial myofibroblasts display an increased expression of vascular endothelial growth factor (VEGF) and hepatocyte growth factor/scatter factor (HGF/SF), which promote EC migration [385].

Glucocorticoids (GCs) are steroidal ligands of the glucocorticoid receptor (GR), which belongs to the nuclear receptor superfamily. Stimulation of GR regulates many physiological processes, mainly via gene transactivation or transrepression [188]. Consequently, glucocorticoids are clinically important as potent anti-inflammatory compounds in treatment of autoimmune diseases [204], and as adjuvants in cancer therapy [348]. Moreover, GCs provide an effective treatment of infantile hemangiomas [276]. GC-mediated inhibition of angiogenesis is well-

documented [333] and has therapeutic potential in the treatment of cancer [287,336]. The direct, growth-inhibitory influence of GCs on vascular smooth muscle cells is well-established [386,387]. Furthermore, more recent investigations have demonstrated GR-dependent, GC-mediated inhibition of tube-like structure formation by ECs *in vitro*, independent of GCs' anti-inflammatory actions [344]. GCs can also inhibit angiogenesis indirectly by suppression of pro-angiogenic factors, such as VEGF and IL-8, produced by prostate cancer cells [287], and possibly by extracellular matrix degradation or modification of cytokine production [388].

We recently reported that GCs regulate CAFs, decreasing production and secretion of a number of factors linked to cancer progression and invasion: tenascin C (TNC), TGFβ, HGF/SF [8,77,389]. These factors are all known to also affect the angiogenic response through a number of mechanisms [101,390,391]. Combined with our data, these studies suggest that GCs could have the ability to inhibit CAF-induced stimulation of angiogenesis by altering the composition of the CAF-derived secretome. Therefore, this investigation addressed the hypothesis that exposure of colon cancer-derived CAFs to GCs can reduce secretion of angiogenic factors and thus inhibit their ability to promote pro-angiogenic changes in ECs.

2.2. Materials and Methods

2.2.1. Cells and reagents

Human colon cancer-derived CAFs (CT5.3hTERT cells) were isolated as described [77,171] and cultured (37°C, 10% CO_2) in Dulbecco's modified Eagles Medium (DMEM; Life Technologies, Merelbeke, Belgium) supplemented with 10% fetal calf serum (Greiner bio-one, Wemmel, Belgium), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Life Technologies). Primary human umbilical vein endothelial cells (HUVEC; Promocell, Heidelberg, Germany) and human aortic endothelial cells (HAoEC; Promocell) were cultured in Endothelial Cell Growth Medium-2 (EGM2; Lonza, Wokingham, UK), containing all manufacturer-supplied supplements (2% FCS, 0.1% VEGF, 0.4% hFGF-2, 0.1% R3-IGF-1, 0.1% hEGF, 0.1% ascorbic acid, 0.1% heparin, 0.1% GA-100) except hydrocortisone. HUVECs were cultured (37°C, 5% CO_2) on 0.1% gelatin-coated flasks and were studied between passages 2 and 7. In experiments we used EGM2 containing 2%FCS or 0%FCS, abbreviated respectively $EGM2^{S+}$ and $EGM2^{S-}$.

Dexamethasone (Dex), hydrocortisone (Hcrt), prednisolone (Pred), fluocinolone acetonide (FA) and the GR antagonist RU38486 (RU) were purchased from Sigma-Aldrich (Diegem, Belgium). All reagents were dissolved in ethanol and used at a final concentration of 1μM, except RU (2μM). A selective GR modulator (SEGRM), compound A (CpdA) was prepared as previously described [268]

and used at a final concentration of 10µM. The total solvent concentration (maximally 0.1%) was consistent in all conditions.

2.2.2. Conditioned medium preparation

Conditioned medium (CM) was obtained from $10x10^6$ CT5.3hTERT CAFs and prepared as described [77]. Briefly, cells were washed three times with serum-free DMEM and treated for 48h with solvent (ethanol), Dex (1µM), Hcrt (1µM), Pred (1µM), CpdA (10µM) or RU (2µM) in serum-free DMEM. After this incubation CM was collected, concentrated 10-fold using centrifugal filter tubes with a 3kDa cut-off (Amicon Ultra, Merck Millipore, Darmstadt, Germany), filter-sterilized (0.2µm pore size) and stored (-20°C) for subsequent functional and biochemical assays. For functional assays CM from solvent and Dex-treated CAFs (CM^{CTRL} and CM^{DEX}, respectively) were diluted with $EGM2^{S+}$ or $EGM2^{S-}$ or with serum-free DMEM prior to treatment. Taking into account the concentrating procedure of CM and further dilution in the functional assays, the maximal final concentration of Dex in the CM^{DEX} treatment was calculated to be 50nM. CM concentrations and dilutions used in particular experiments are listed in Addendum 2 (Supplementary Table 1).

2.2.3. Protein analysis: protein array, western blot and immunoassay (ELISA)

CM from CT5.3hTERT CAFs treated with Dex or solvent (CM^{CTRL} and CM^{DEX}, respectively) were collected after 48h, 4-fold concentrated and subjected to Ray Bio® Biotin Label-based Human Antibody Array I (Raybiotech, GA, USA, cat no: AAH-BLM-I-2) which allows simultaneous analysis of expression levels of 507 human target proteins (including cytokines, chemokines, adipokines, growth factors, angiogenic factors, proteases, soluble receptors and soluble adhesion molecules) in cell culture supernatants. The assay was performed according to the manufacturer's instructions with the results visualized using X-Ray films (GE Healthcare, Diegem, Belgium) and the signal evaluated using ImageJ software [362]. For further analysis, we set the threshold value for the ratio between relative protein signals in CM^{CTRL} vs. CM^{DEX} as greater than 1.5. Selected factors analyzed using the protein array are listed in Addendum 2 (Supplementary Table 2).

For further validation of the protein array results, CT5.3hTERT CAFs were incubated for 48h with steroids (Dex, Hcrt, Pred; 1µM), CpdA (10µM), RU (2µM) or solvent. Conditioned media were collected, concentrated (10-fold) and protein concentrations were evaluated using the Lowry method [392]. Samples were prepared in SDS sample buffer (50 mM Tris pH 6.8; 2% SDS; 10% glycerol; bromophenol blue; 100 mM DTT), loaded (25µg) onto an SDS-PAGE gel and subjected to the standard Western Blot protocol, as described by Santa Cruz (Santa Cruz, Heidelberg, Germany). The proteins were probed using the following primary anti-human antibodies: anti-uPA (H-140) (1/500, Santa Cruz Biotechnology, cat no: sc-14019), anti-ANG I (H-123) (1/500, Santa Cruz Biotechnology, cat no: sc-9044) and anti-ANGPTL2 (P-13) (1/500, Santa Cruz Biotechnology,

cat no: sc-107143). Results were visualized using species-specific HRP-linked secondary antibodies and reagents: anti-rabbit (1/4000, GE Healthcare, cat no: NA934V), anti-goat (1/3000, Santa Cruz Biotechnology, cat no: sc-2020), ECL solution (Thermo Scientific, Gent, Belgium) and X-Ray films (GE Healthcare). Signal quantifications were performed using ImageJ software [362].

The internalization and subsequent degradation of the acetylated low density lipoprotein (Ac-LDL) is a characteristic feature of endothelial cells. In order to evaluate whether the conditioned medium from CAFs affects the basic endothelial character of HUVECs, we performed an Ac-LDL uptake assay. Briefly HUVECs were incubated for 24h in $EGM2^{S+}$ (control), DMEM, CM^{CTRL} or CM^{DEX}. DMEM and 10-fold concentrated CM were diluted 1:1 with $EGM2^{S+}$. An Ac-LDL assay was then performed, as described in Supplementary methods (Addendum 2).

In order to determine the concentrations of prostanoids in conditioned medium from CAFs and HUVECs, and in HUVEC lysates, we performed immunoassays (ELISAs) for prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostacyclin (PGI_2; by assessing 6-keto-$PGF_{1\alpha}$) and prostaglandin E2 (PGE_2), according to manufacturer's instructions (Enzo Life Sciences, Antwerp, Belgium, cat no: ADI-900-069, ADI-900-001 and ADI-900-004, respectively). Absorbance was quantified on Paradigm Detection Platform (Beckman Coulter) using SoftMax Pro 6.1 software. HUVEC lysates were prepared from cells treated with $EGM2^{S+}$ (control), CM^{CTRL} or CM^{DEX} (diluted 1:1 with $EGM2^{S+}$, giving a final 5-fold concentration of CM). After 24h cells were lyzed with TOTEX buffer (20mM Hepes/KOH pH 7.9; 0.35M NaCl; 20% glycerol; 1% NP40; 1mM $MgCl_2$; 0.5mM EDTA; 0.1mM EGTA; 1/100 HALT Protease and Phosphatase Inhibitor Cocktail, ThermoFisher scientific, cat no: 78440) and the lysates were subjected to immunoassays.

2.2.4. RNA isolation and RT-qPCR

CT5.3hTERT CAFs were incubated for 48h with steroids (Dex, Hcrt, FA, Pred; 1µM), CpdA (10µM), RU (2µM) or solvent (control). HUVECs were incubated for 24h with $EGM2^{S+}$ (control), DMEM, CM^{CTRL} or CM^{DEX}. DMEM and CM were diluted 1:1 with $EGM2^{S+}$, the final CM concentration was 5-fold. To isolate the total RNA from CAFs we used TRIzol reagent (Life Technologies) and to isolate HUVEC RNA we used an RNeasy Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Reverse transcription (RT) of CAF RNA was performed using the iScript kit (Bio-Rad, Temse, Belgium), while RT of HUVEC RNA was performed using QuantiTect Reverse Transcription Kit (Qiagen). The cDNA obtained was subjected to quantitative PCR (qPCR) using LightCycler 480 SYBR Green I Master reagents (Roche Diagnostics, Rotkreuz, Switzerland), according to the manufacturer's instructions. qPCR reactions were performed in triplicate using the LightCycler 480 system (Roche Diagnostics), with the following conditions: (A) initial denaturation 95 °C, 5min; (B) 45 cycles of denaturation 95°C, 15s, annealing and elongation 60 °C, 45s. Primer

sequences are listed in Addendum 5 (Supplementary Table 4). Specific signal of the gene of interest was normalized to the respective geometric mean expression level of 3 reference genes (GAPDH, PPIB, 36B4).

2.2.5. Cell viability (MTT) and proliferation (SRB) assays

To test viability HUVECs were seeded in 96-well plates, equilibrated in $EGM2^{S+}$ for 24h and incubated for 24h with DMEM, CM^{CTRL} or CM^{DEX}. DMEM and 10-fold concentrated CM were diluted 1:1 with $EGM2^{S+}$. As a negative control HUVECs were treated with 10% Triton X-100 (Sigma-Aldrich) for 1h (data not shown). Cell viability was assessed using a classic 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [393], performed with reagents purchased from Sigma-Aldrich.

Proliferation was assessed using a sulforhodamine-B (SRB) test, as described previously [394]. HUVECs seeded in 96-well plates were left to equilibrate in $EGM2^{S+}$ for 24h and then incubated in $EGM2^{S+}$ or $EGM2^{S-}$, DMEM, CM^{CTRL}, CM^{CTRL}+Dex (50nM) or CM^{DEX} for 24-72h. DMEM and 10-fold concentrated CM were diluted 1:1 with $EGM2^{S+}$ or $EGM2^{S-}$. Results were obtained using a Molecular Devices OPTImax Microplate Reader and the SoftMax® Pro 3.0 software. Data were expressed on a scale where maximal proliferation in controls ($EGM2^{S+}$ at 72h) was set to 100%.

2.2.6. Scratch assay

HUVEC migration was assessed using the IncuCyte ZOOM Scratch assay (Essen Bioscience, Hertfordshire, UK) according to manufacturer's instructions. Briefly, $3x10^4$ HUVECs/well were seeded in 96-well culture plates and cultured for 18h in $EGM2^{S+}$ at 37°C, 5% CO_2. A scratch was then made using the WoundMaker tool (Essen Bioscience). The cells were washed twice with medium, and the medium was then replaced with $EGM2^{S+}$, $EGM2^{S-}$, CM^{CTRL}, CM^{CTRL}+Dex (50nM) or CM^{DEX}. 10-fold concentrated CM were diluted 1:1 with $EGM2^{S-}$. Plates were then installed in the IncuCyte ZOOM system and images (10x magnification) of the wound were recorded in each well every hour for 48h. Scratch closure rate was evaluated with the IncuCyte software, expressed as percentage of relative wound density (RWD) over a 30h period. RWD = 0 at time 0 and 100% when cell confluence within the wound area is equal to that outside the initial wound area, thus normalizing for changes in cell density due to proliferation or other non-motogenic pharmacological effects. The area under the curve (AUC) was calculated for each condition and the results are expressed as AUC from RWD.

2.2.7. Tube-like structure (TLS) formation assay

The TLS assay was performed by seeding HUVECs or HAoECs onto Matrigel™, as previously described [395]. Briefly, HUVECs and HAoECs ($15x10^3$ cells/well) were seeded in 96-well plates coated with Matrigel™ matrix (Corning, Flintshire, UK) in either $EGM2^{S+}$, DMEM, CM^{CTRL} or CM^{DEX}. DMEM and 10-fold concentrated CM were diluted 1:4 with $EGM2^{S+}$. This assay required a lower concentration of CM than that used in other experiments (4:1 ratio $EGM2^{S+}$:CM, giving a final concentration of 2x basal CM) as ECs failed to generate TLS networks when $EGM2^{S+}$ was used in 1:1 ratio with DMEM. Phase-contrast images (5x magnification) of the centre of each well were taken 3h, 6h and 23h post induction and TLS formation was evaluated using the Angiogenesis Analyzer plug-in developed for the ImageJ software [362] by Carpentier *et al.* [396]. The total length of tubes, number of junctions, and number of segments were calculated from images taken when the network reached stability (6h post induction for HUVECs; 3h post induction for HAoECs).

2.2.8. Aortic ring assay

For the *ex vivo* aortic ring assay [397] C57BL/6 male mice aged 8-12 weeks (Charles River Laboratories) were sacrificed by CO_2 asphyxiation at day 0 and the thoracic aortas were isolated and washed with serum-free DMEM. Isolated aortas were cleaned of connective tissue, divided into 1-2 mm rings and embedded in rat tail collagen type 1 (1mg/ml, Sigma-Aldrich). Rings were then incubated (37°C, 5% CO_2) in serum-free DMEM (control), CM^{CTRL}, CM^{CTRL}+Dex (50nM) or CM^{DEX}. 10-fold concentrated CM were diluted 1:1 with serum-free DMEM. Media were replaced after 3 and 7 days in culture. Phase-contrast microscopy was used to count outgrowths on days 5, 7 and 10. Phase-contrast images (5x magnification) were taken at the corresponding time points. Sprout lengths were measured on pictures obtained after 10 days post treatment using ImageJ software [362]. Higher power images of formed sprouts are displayed in Addendum 2 (Supplementary Figure 8).

2.2.9. Statistical analyses

Data are presented as mean ± standard deviation or as a Tukey's box plot. Statistical analysis was performed using GraphPad Prism 5.03 with a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test, or with Mann-Whitney U test, as appropriate. The applied test is indicated in the figure legends. A $p < 0.05$ was considered statistically significant. Where applicable, results were expressed as a relative number and the untreated condition was set as 1, 100 or 100% and other conditions were recalculated accordingly.

2.3. Results

2.3.1. Glucocorticoids modify secretion of angiogenic factors by CAFs.

In order to obtain a broader insight into the effects of GR modulation on colon cancer-derived CAFs, we performed a protein array which detects over 500 different proteins from cell supernatants. Analysis of the protein array data (Figure 26A, Supplementary Figure 3) indicated that incubation with Dex (1µM) for 48 hours reduced the expression of urokinase-type plasminogen activator (uPA) and angiopoietin-like protein-2 (ANGPTL2), but increased expression of angiogenin (ANG) in supernatant from CT5.3hTERT CAFs. The array also identified a number of factors present in the CM from CAFs that were not sensitive to Dex treatment. Selected angiogenesis-related and inflammatory factors are listed in Addendum 2 (Supplementary Table 2). Western blot analyses (Figure 26B) and RT-qPCRs (Figure 26C-E), were used to verify the results obtained from the protein array and to possibly extend our findings to other GR ligands and modulators. Western blot analysis of 10-fold concentrated cell supernatants confirmed that Dex (1µM; 48h) reduced protein levels of uPA, and ANGPTL2, whilst increasing ANG protein levels (Figure 26B). A similar regulation pattern was seen with other glucocorticoids (Hcrt and Pred). In contrast, the SEGRM CpdA (10µM; 48h) did not reduce uPA and ANGPTL2 protein levels and did not upregulate ANG. RU (2µM; 48h) alone had no effect, but blocked Dex-induced changes which suggests a GR-regulated mechanism. RT-qPCR of mRNA isolated from CT5.3hTERT cells showed that glucocorticoids seemed to reduce expression of uPA (Figure 26C) and ANGPTL2 (Figure 26D), but this only achieved significance for the effects of Dex and Pred on ANGPTL2. The length of exposure to Dex matters here, as Dex-induced reduction of uPA expression was found to be significant after a shorter (6h) exposure (Addendum 2, Supplementary Figure 4). Consistently, all glucocorticoids significantly upregulated ANG (Figure 26E). In contrast, to what is observed for protein, CpdA yielded different results at the transcriptional level, following 48h of treatment, and upregulated the mRNA levels of uPA, ANGPTL2 and ANG. Treatment with RU had no effect on ANG and ANGPTL2 mRNA levels, but dramatically increased expression of uPA. Any effect of Dex was lost or reduced in the presence of RU (Figure 26C-E).

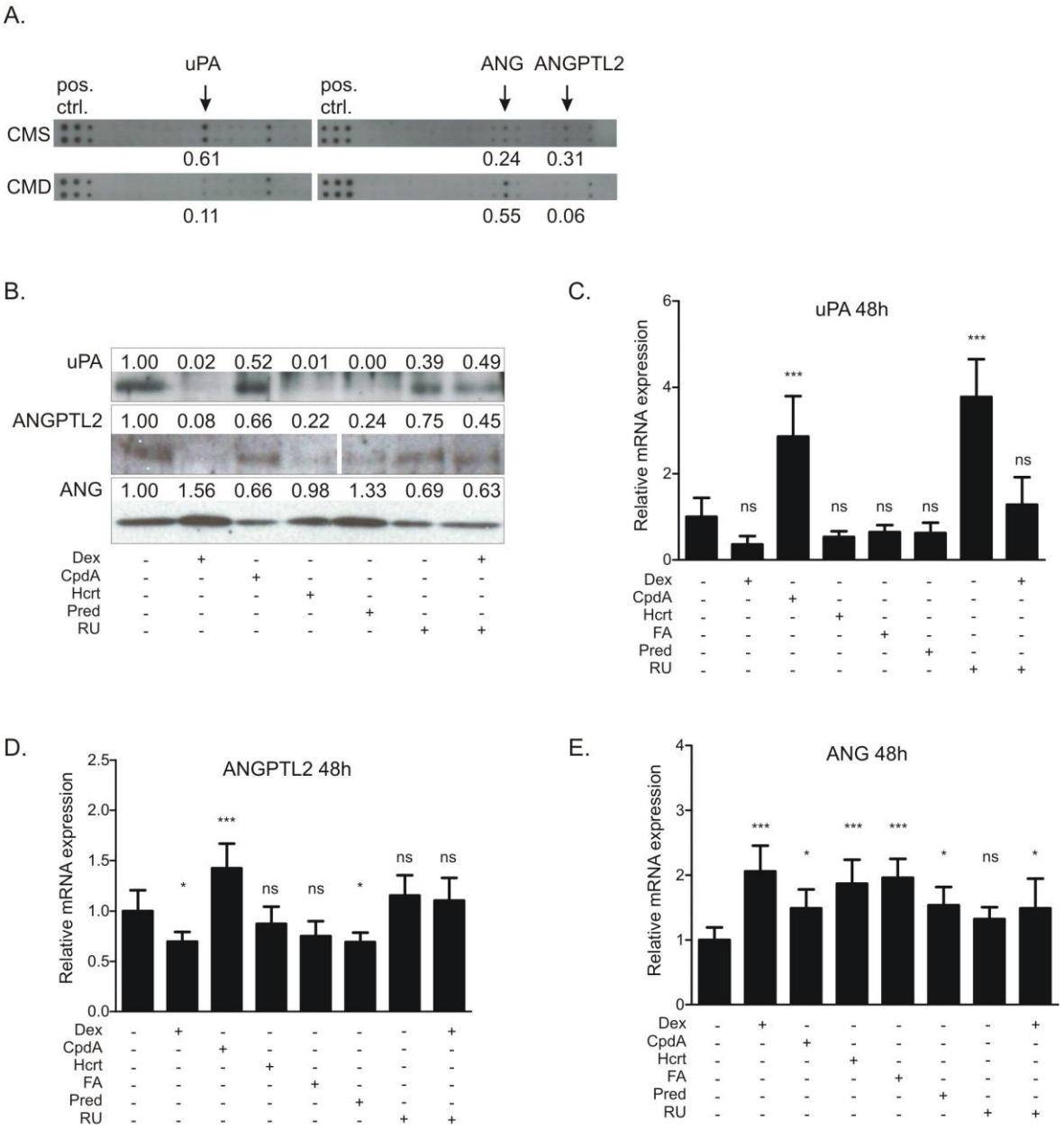

Figure 26. Glucocorticoids alter the secretion of proteins implicated in angiogenesis from cultured CAFs. (A) CT5.3hTERT cells were treated with solvent or Dex (1µM). After 48h cell supernatants were collected, 4-fold concentrated and subjected to Ray Bio® Biotin Label-based Human Antibody Array I. Relevant fragments of the array are displayed. The six dots displayed on the left of the array act as a positive control (pos.ctrl.). (B) CT5.3hTERT cells were treated with solvent, Dex (1µM), CpdA (10µM), Hcrt (1µM), Pred (1µM), or RU (2µM) or co-treated with Dex (1µM) and RU (2µM) for 48h. Cell supernatants were collected, 10-fold concentrated and subjected to Western Blot analysis for the detection of uPA, ANG and ANGPTL2. Protein bands representing ANGPTL2 belong to the same blot. Results are representative of three independent experiments. (A, B) Western blot and protein array signals were quantified using ImageJ software [362]. (C, D, E) CT5.3hTERT cells were treated for 48h with solvent, Dex (1µM), CpdA (10µM), Hcrt (1µM), FA (1µM), Pred (1µM), RU (2µM) or co-treated with Dex (1µM) and RU (2µM). mRNA isolated from cells was subjected to RT-qPCR assaying uPA, ANG and ANGPTL2 mRNA levels. Results were normalized to the respective geometric mean of GAPDH, PPIB and 36B4 reference genes' mRNA levels. Results are shown as the mean ± SD of three independent experiments and statistical analysis was performed using a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test. ns: not significant, *: $p<0.05$, ***: $p<0.001$.

2.3.2. Conditioned medium from dexamethasone-treated CAFs contains decreased levels of prostanoids.

Prostanoids are known to influence cell proliferation and migration. Immunoassays (ELISAs) demonstrated that $PGF_{2\alpha}$, PGI_2 (by assessing 6-keto-$PGF_{1\alpha}$) and PGE_2 were all present in conditioned medium from solvent-treated CAFs (CM^{CTRL}) (Figure 27). There was a pattern of decreased levels of all three prostanoids in conditioned medium from CAFs exposed to dexamethasone (CM^{DEX}), which achieved significance for $PGF_{2\alpha}$ (Figure 27A) and PGI_2 (Figure 27B) but not for PGE_2 (Figure 27C).

ELISA demonstrated that $PGF_{2\alpha}$, PGI_2 and PGE_2 were also present in medium from HUVECs (Addendum 2, Supplementary Figure 4A-C). Exposure to CM^{CTRL} (24h) did not alter the concentration of these prostanoids in HUVEC supernatants and this response was not altered if the conditioned medium was derived from Dex-treated CAFs (CM^{DEX}). These prostanoids were also detected in HUVEC lysates but their concentrations were not altered by 24h exposure to CM^{CTRL} or CM^{DEX} (Addendum 2, Supplementary Figure 5D-F).

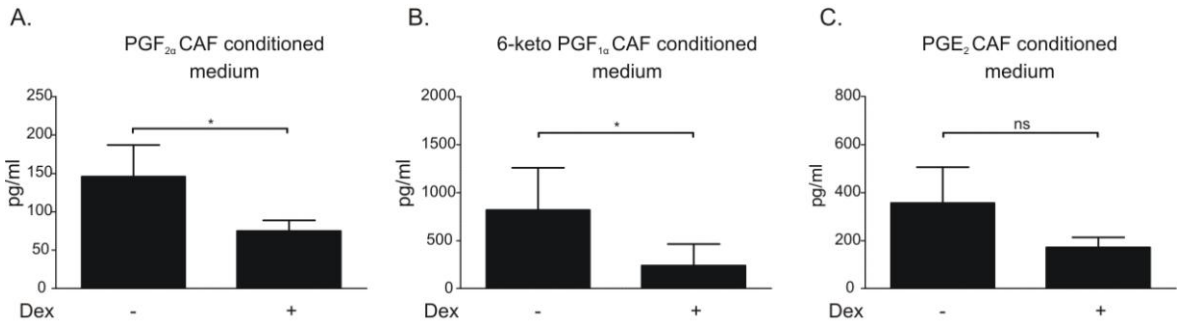

Figure 27. Conditioned medium from dexamethasone-treated CAFs contains reduced levels of prostanoids. CT5.3hTERT cells were treated with solvent or Dex (1μM). After 48h cell supernatants were collected, 10-fold concentrated and analyzed (ELISA) for (A) $PGF_{2\alpha}$, (B) PGI2 (by assessing 6-keto-$PGF_{1\alpha}$) and (C) PGE_2 levels. Results are the mean ± SD of four independent experiments and statistical analysis was performed using a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test, ns: not significant, *: $p<0.05$.

2.3.3. HUVEC proliferation is promoted by conditioned medium from CAFs.

The impact of conditioned medium from CAFs on the basic endothelial character of HUVECs was assessed using an Ac-LDL uptake assay. Results indicate Ac-LDL uptake was not altered in HUVECs following 24h exposure to DMEM, CM^{CTRL} or CM^{DEX} (Addendum 2 Supplementary Figure 6).

Prior to proliferation experiments, an MTT assay was performed and demonstrated no negative effect on viability or metabolism from either treatment (Figure 28A). On the contrary, CM^{CTRL} and CM^{DEX} treatment increased the production of the MTT formazan product.

In order to investigate the impact of CAF CM on EC growth we performed an SRB assay. In comparison with the control treatment ($EGM2^{S+}$) HUVEC proliferation was reduced by exposure to

DMEM (Figure 28B). However, proliferation was increased compared with $EGM2^{S+}$ when the HUVECs were exposed to CM^{CTRL} after 48 and 72h. Use of CM^{DEX} did not result in a significant difference with the $EGM2^{S+}$ control.

In the absence of FCS (Figure 28C), both CM^{CTRL} and CM^{DEX} induced a dramatic increase in HUVEC proliferation compared with $EGM2^{S-}$ control, at 24h and 72h post treatment. Addition of Dex (50nM) to CM^{CTRL} did not alter HUVEC proliferation.

2.3.4. Conditioned medium from solvent-treated CAFs causes an increase in HUVEC migration which is lost with conditioned medium from Dex-treated cells.

One of the crucial events of angiogenesis includes EC migration into perivascular stroma, due to the presence of pro-angiogenic factors. In the scratch assay (Figure 29, Addendum 2, Supplementary Figure 7), 30h exposure to CM^{CTRL} increased (by approximately 25% compared with EGM^{S-}) the area under the curve (AUC) (Figure 29C), indicating accelerated wound healing. This acceleration was not seen when cells were exposed to CM^{DEX} (Figure 29B, C). These data suggest, therefore, that CM^{CTRL} contains a factor(s) that stimulate HUVEC migration which is not present in CM^{DEX}. Direct addition of Dex (50nM) did not abolish the CM^{CTRL}-induced increase in wound healing (Figure 29B, C), indicating the lack of effects with CM^{DEX} was not due to residual Dex.

2.3.5. Conditioned medium from CAFs does not influence tube-like structure formation by HUVECs or HAoECs.

The ability of ECs to form three-dimensional structures (tube-like structures, TLS) represents cell differentiation belonging to a later phase of angiogenesis (Addendum 2, Supplementary Figure 8). The ability of HUVECs to form a net of TLS was mildly impaired after incubation with DMEM, CM^{CTRL} or CM^{DEX} diluted in a ratio of 1:4 with $EGM2^{S+}$, as compared to untreated control ($EGM2^{S+}$). This inhibition only achieved significance for total tubule length (Figure 30A), but not for number of junctions (Figure 30B) or number of segments (Figure 30C). Exposure to conditioned media had a similar effect in HAoECs with neither CM^{CTRL} nor CM^{DEX} significantly altering total tubule length (Figure 30D), number of junctions (Figure 30E) or number of segments (Figure 30F).

2.3.6. Conditioned medium from CAFs alters gene expression in HUVECS.

In order to investigate whether the CAF-derived CM affects the expression of angiogenesis-related genes in HUVECs, we performed RT-qPCR for VEGF, VEGFR1, VEGFR2 and IL-6. RT-qPCR was run on mRNA obtained from HUVECs exposed for 24h to DMEM or conditioned medium, diluted in a 1:1 ratio with $EGM2^{S+}$. Exposure to CM produced an apparent reduction in VEGF mRNA expression (Figure 31A) that achieved significance (0.62-fold change) only for the comparison of CM^{CTRL} with the untreated ($EGM2^{S+}$) control. CM had little effect on VEGFR1 (Figure 31B) or VEGFR2 (Figure

31C) mRNA, with a small increase (1.63 fold change) in VEGFR1 seen only when CM^{DEX}-treated cells were compared with untreated ($EGM2^{S+}$) controls. In contrast, exposure to CM^{CTRL} induced a clear (2.16-fold) increase in IL-6 transcripts (Figure 31D) which was not observed when cells were exposed to CM^{DEX}.

2.3.7. Conditioned medium from CAFs promotes outgrowth formation from mouse aortic rings.

The organ culture-aortic ring assay enabled investigation of the effects of CAF-derived conditioned medium in a more complex model of angiogenic tube formation, involving the presence of non-endothelial cells (smooth muscle cells, fibroblasts, pericytes, inflammatory cells) in an intact arterial ring (rather than in 2 dimensional culture). *Ex vivo* outgrowth vessel formation in mouse aortic rings (Figure 32) was increased by exposure to CM^{CTRL} after 5 days (Figure 32A), 7 days (Figure 32B) and 10 days (Figure 32C) of incubation, compared to untreated control (DMEM). This effect was less pronounced using CM^{DEX} and by addition of Dex (50nM) to CM^{CTRL}. In addition, the length of outgrowths (Addendum 2, Supplementary Figure 9) was reduced by exposure to CM^{DEX} or by addition of Dex (50nM) to CM^{CTRL} (Figure 33).

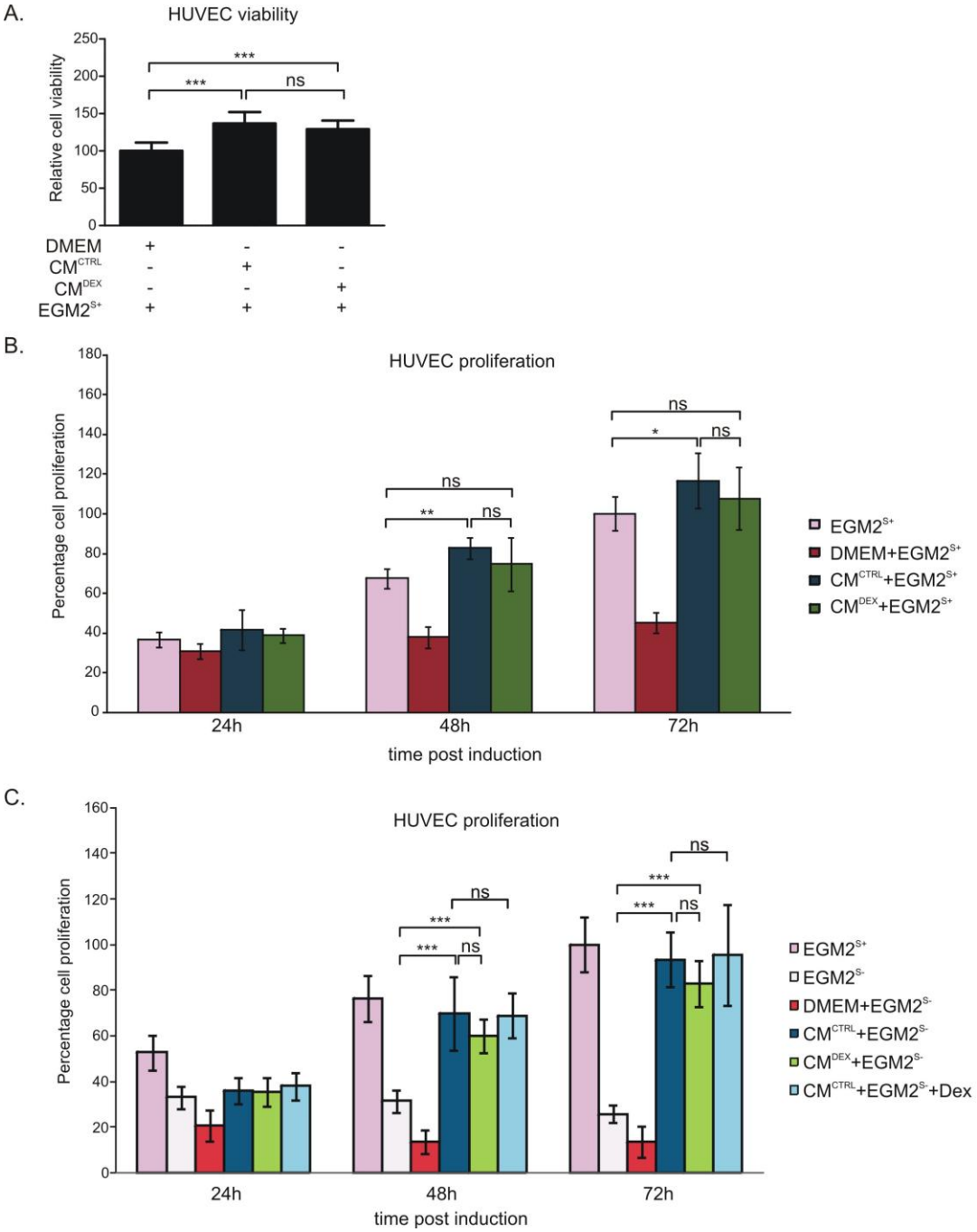

Figure 28. HUVEC proliferation is promoted by conditioned medium from CAFs. (A) HUVECs were treated with $EGM2^{S+}$ mixtures with DMEM, CM^{CTRL} or CM^{DEX} in 1:1 ratio. After 24h cells were subjected to MTT assay and percentage cell viability was assessed. Obtained values were normalized to the values obtained from cells treated with DMEM and other conditions were recalculated accordingly. (B) HUVECs were treated with either $EGM2^{S+}$ (control) or with $EGM2^{S+}$ mixtures with DMEM, CM^{CTRL} or CM^{DEX} in 1:1 ratio. After 24h, 48h and 72h cells were subjected to SRB assay and percentage cell proliferation was calculated. Obtained values were normalized to a control of untreated cells at 72h, which indicates their maximal proliferation. (C) HUVECs were treated with either $EGM2^{S+}$, $EGM2^{S-}$ or with $EGM2^{S-}$ mixtures with DMEM, CM^{CTRL}, CM^{DEX} or CM^{CTRL}+Dex (50 nM) in 1:1 ratio with $EGM2^{S-}$. Obtained values were normalized to a control of cells treated with $EGM2^{S+}$ at 72h, which indicates their maximal proliferation. Results (A, B, C) are the mean ± SD of at least three independent experiments and statistical analysis was performed using a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test. ns: not significant, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

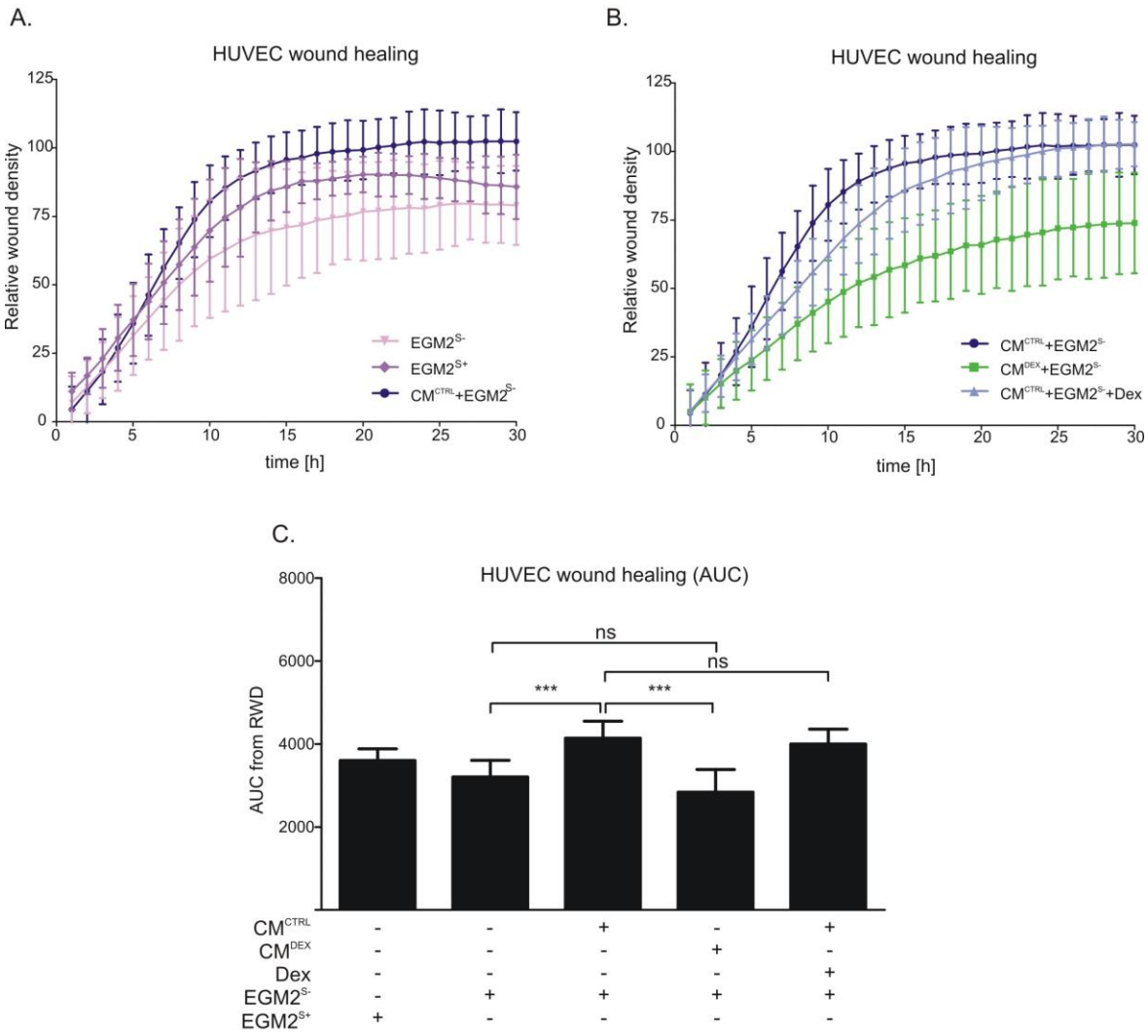

Figure 29. Acceleration of HUVEC migration by CAF-derived conditioned medium is lost when the CAFs are exposed to dexamethasone. (A, B, C) HUVECs were cultured in $EGM2^{S+}$. After 18h a wound was created in the confluent cell monolayer. Cells were washed and treated with either $EGM2^{S+}$, $EGM2^{S-}$, or $EGM2^{S-}$ mixtures with CM^{CTRL}, CM^{DEX} or CM^{CTRL}+Dex (50 nM) in 1:1 ratio. (A, B) The wound healing process was examined with the IncuCyte ZOOM system, measuring percentage relative wound density (RWD) for each condition every hour. (C) Area under curve (AUC) was calculated for each treatment and displayed in parallel. Results (A, B, C) are represented as the mean ± SD of four independent experiments and statistical analysis was performed using a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test. ns: not significant, ***: $p<0.001$.

2.4. Discussion

This investigation addressed the hypothesis that exposure of CAFs to glucocorticoids would reduce secretion of angiogenic factors and inhibit their ability to promote angiogenesis. We showed that conditioned medium from colon cancer-derived CAFs stimulated proliferation and migration of HUVECs. Secretion of certain angiogenic factors was altered in conditioned medium from Dex-treated CAFs (CM^{DEX}), and this was associated with a reduced HUVEC migration, but did not affect HUVEC proliferation. Exposure to conditioned media only slightly altered expression of angiogenic genes in HUVECs, and had no effect on tube-like structure formation in a 2-

dimensional assay (with HUVECS and HAoECs). In contrast, in an *ex vivo* model (mouse aorta), conditioned media from CAFs increased the number and length of vascular outgrowths. This effect was impaired when CAFs had been exposed to Dex; most likely a combined result of direct inhibition by residual steroid in the conditioned medium together with Dex-driven reduction of certain factors secreted by CAFs.

Angiogenesis is a complex, multi-step process regulated by a balance between pro- and anti-angiogenic factors [85,96]. It can be modified at various stages, including degradation of basement membrane and EC shape change, invasion, migration and proliferation of ECs to form a migrating column, EC differentiation, formation of tight connections and capillary tubes, fusion with other vessels and cell maturation and pruning [12]. CAFs have the potential to regulate angiogenesis during tumor development. They are recruited by cancer cells and act as potent promoters of tumor growth and invasion [76]. For example, breast cancer-associated fibroblasts promote tumor microvascularization, leading to enhanced tumor growth [139]. CAF-mediated regulation of vessel formation in cancer could be attributed to direct and/or indirect modulation of angiogenesis [76,138]. We have previously shown the GC-sensitive and GR-regulated release of several pro-angiogenic factors (TNC, TGFβ and HGF/SF) by cultured colon cancer-derived CAFs [389]. The present study extends this work by showing that these cells secrete factors that promote survival, proliferation and migration of ECs. In culture conditions devoid of serum, CM from colon cancer-derived CAFs could compensate for the absence of FCS, thus maintaining HUVEC proliferation (Figure 28C) and migration (Figure 29A). A similar increase in HUVEC migration was demonstrated with CM collected from intestinal sub-epithelial 18Co myofibroblasts, an effect that was stimulated by pre-treatment of the myofibroblasts with prostaglandin (PG)E_2 [385].

Since CAF-derived CM regulated migration and proliferation of ECs, it was reasonable to propose that it might also influence angiogenesis through direct interaction with the endothelium. However, the failure of CM to stimulate TLS formation by HUVECs or HAoECs in a 2-dimensional assay suggests that this was not the case (Figure 30). Furthermore, a lack of effect on angiogenesis is consistent with the relatively small impact of CM on expression of angiogenic factors in HUVECs (Figure 31). VEGF is a key promoter of angiogenesis, and acts through interaction with VEGFR2 [45,96]. VEGFR1, which has a 10-fold weaker kinase activity than VEGFR2, may act as a VEGF-trap and, thus, suppress angiogenesis [45,95]. IL-6 is a multifunctional pro-inflammatory cytokine which has potent pro-angiogenic properties [398,399]. Interestingly, CM from intestinal subepithelial 18Co myofibroblasts were also ineffective unless activated with PGE_2, which increased VEGF expression in those myofibroblasts [385]. Similarly, mouse embryonic fibroblasts did not promote tube formation unless pre-treated with CM from gastric tumor cells,

which increased expression of VEGF in the fibroblasts [400]. These findings suggest that CM obtained from colon CAFs contain insufficient levels of VEGF and, coupled with a lack of VEGF activation in HUVECs treated with CM, could explain the inability of CM to stimulate tube formation by isolated HUVECs and HAoECs.

GCs are exploited clinically, predominantly for their anti-inflammatory properties, for the treatment of numerous disorders, including asthma and rheumatoid arthritis [204]. They also serve as adjuvants in tumor therapy [348]. However, the influence of GCs on the solid tumor and its microenvironment is controversial and not fully understood [281]. In prostate and breast cancer GC therapy has some benefits, whereas in gastro-intestinal cancer GC treatment has no effect and in lung cancer may even be detrimental [278]. We have previously shown that production and secretion of TNC, TGFβ and HGF/SF by CAFs is reduced by GC treatment [389]. This is comparable with the demonstration here that GCs reduce expression and secretion of uPA and ANGPTL2, whilst upregulating ANG (Figure 26). This response is probably mediated via a GR-dependent mechanism, since Dex is relatively GR selective and its effects were blocked by GR antagonism with RU. The alterations in uPA, ANGPTL2 and ANG secretion were observed with other GCs, namely Hcrt, FA and Pred. Although the non-steroidal SEGRM CpdA [268] is able to transrepress the expression of several GR-regulated genes in CAFs [389], it suppresses neither uPA nor ANGPTL2 protein levels. As previously reported [268,389] CpdA is unable to transactivate GC-inducible genes via a classic GRE-mediated mechanism. Therefore, consistent with our results, any effect on ANG protein or mRNA level was not expected. These findings support a common mechanism for the action of GCs and add to the evidence that CpdA has different signaling properties than classic GCs [389]. The factors identified as affected by GCs have been linked to cancer progression and/or angiogenesis. uPA regulates vascular remodeling [401] and its expression correlates with tumor angiogenesis and tumor vessel invasion in gastric and breast cancer [402,403]. ANG is named for its ability to stimulate vessel growth, in normal and pathological states, including in tumors [106]. Angiopoietin-like proteins can stimulate vascular cells and influence metabolism and tumor biology [404]. Thus it was logical to predict that modulation of these factors by exposure of the CAFs to Dex might influence angiogenesis. Moreover, levels of prostaglandins, factors known to modulate inflammatory response and to promote cancer progression [405,406], were also reduced by Dex treatment in CAF-derived conditioned medium (Figure 27). This corresponds with previous reports listing glucocorticoids as inhibitors of prostaglandin synthesis [407].

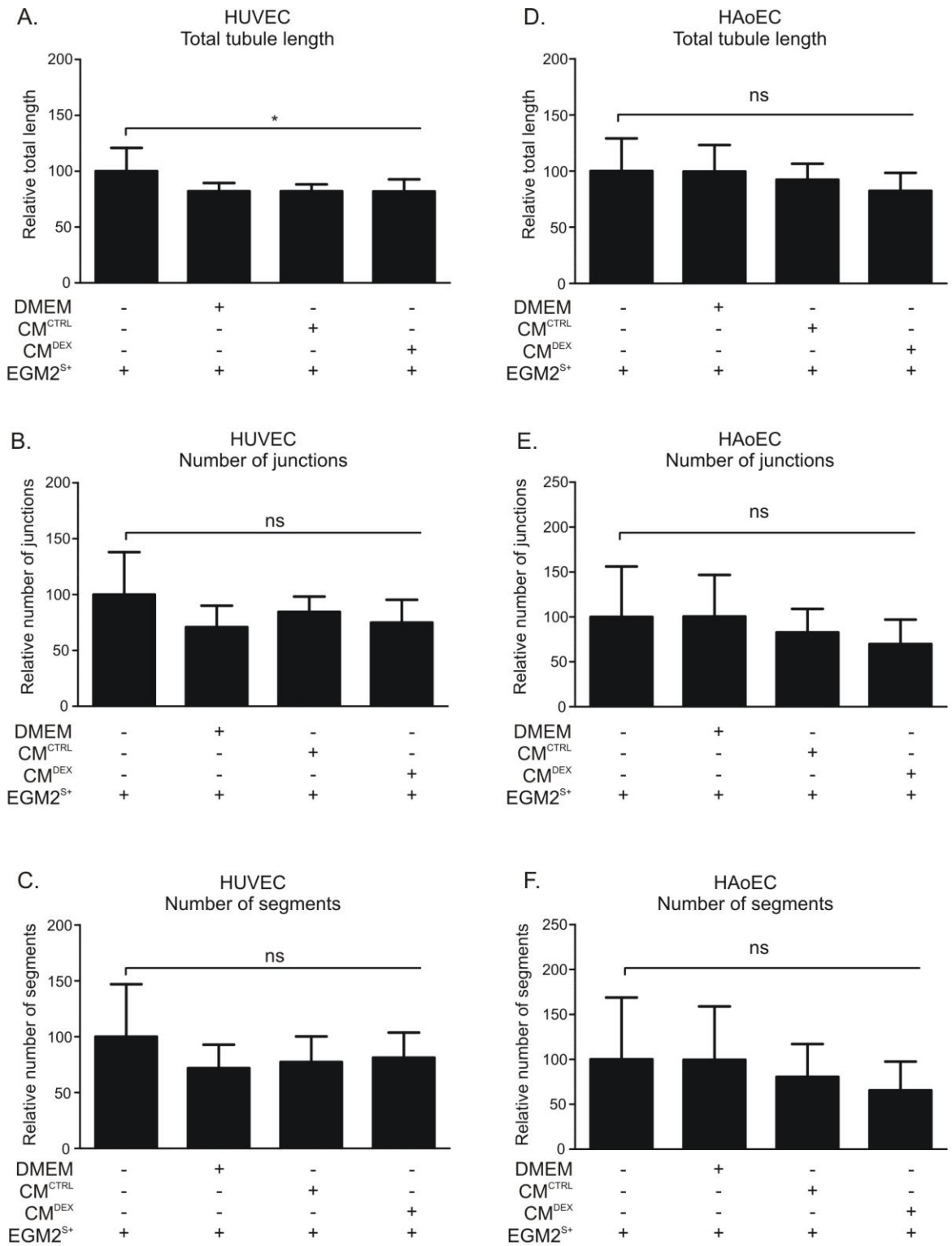

Figure 30. Conditioned medium from myofibroblasts does not affect tube-like structure formation by HUVECs or HAoECs. HUVECs (A, B, C) and HAoECs (D, E, F) were seeded on Matrigel-coated wells and treated with either $EGM2^{S+}$ or $EGM2^{S+}$ mixtures with DMEM, CM^{CTRL} or CM^{DEX} in 1:4 ratio. Phase-contrast images were taken at 6h post induction for HUVECs and 3h post induction for HAoECs. The total tubule length (A, D), number of junctions (B, E) and number of segments (C, F) were assessed using Angiogenesis Analyzer plug-in for ImageJ software [362,396]. Results are the mean ± SD of three independent experiments and statistical analysis was performed using a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test, ns: not significant, *: $p<0.05$.

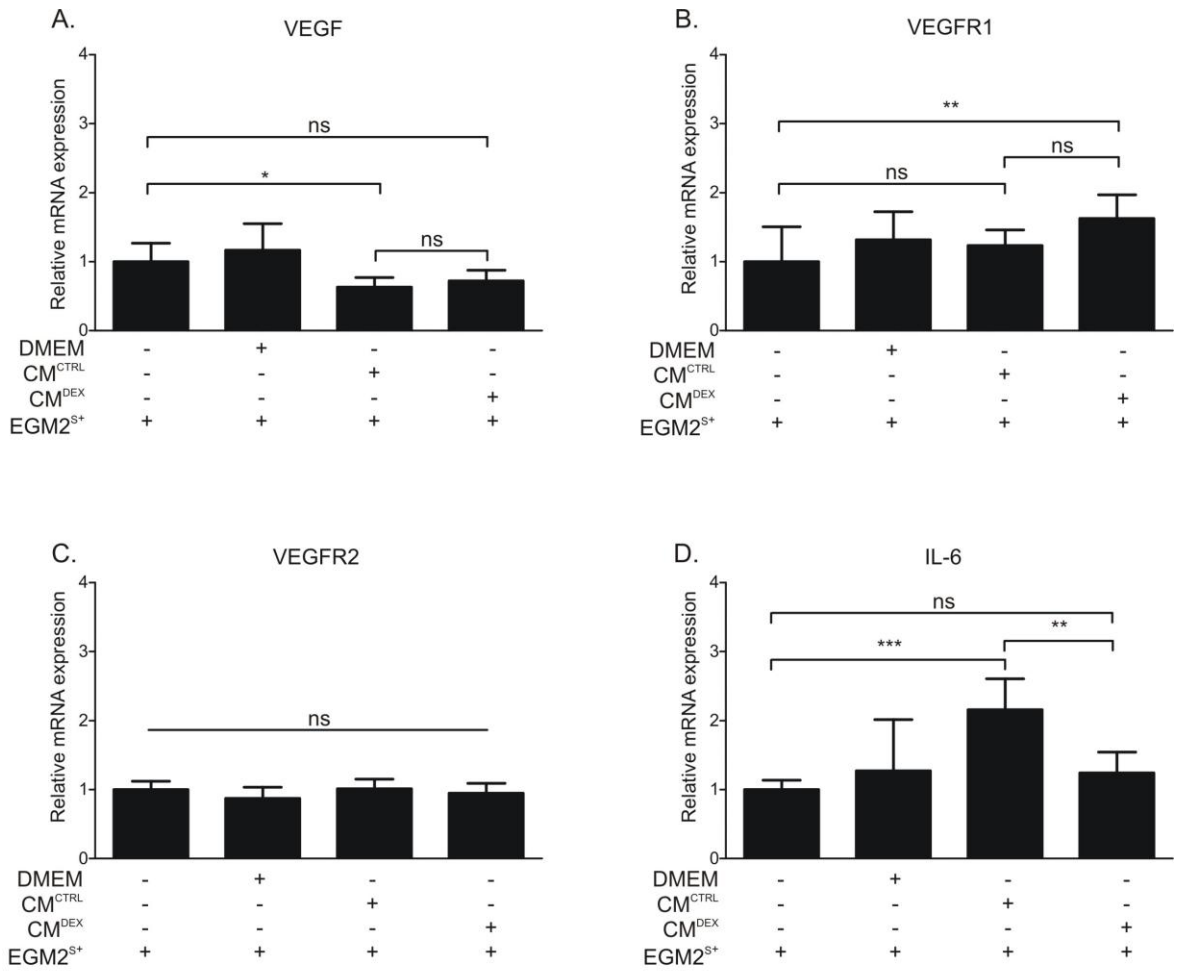

Figure 31. Influence of conditioned medium from CAFs on angiogenesis-related gene expression in HUVECs. (A, B, C, D) HUVECs were treated with either $EGM2^{S+}$ or $EGM2^{S+}$ mixtures with DMEM, CM^{CTRL} or CM^{DEX} in 1:1 ratio. After 24h, mRNA isolated from cells was subjected to RT-qPCR assaying (A) VEGF, (B) VEGFR1, (C) VEGFR2 and (D) IL-6 mRNA levels. Obtained results were normalized to the respective geometric mean of GAPDH, PPIB and 36B4 reference genes' mRNA levels. Results are the mean ± SD of three independent experiments and statistical analysis was performed using a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test. Ns: not significant, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

The failure of CM^{DEX} to alter the CM^{CTRL}-induced stimulation of HUVEC proliferation (Figure 28B, C) indicates that modulation of uPA, ANGPTL2, ANG or prostaglandins does not influence growth of these cells. There was no evidence that CAF-derived conditioned media induce prostaglandin production by HUVECs. This contrasted with the dramatic reduction in HUVEC migration when CM^{DEX} was compared with CM^{CTRL} (Figure 29B, C). The fact that this effect was not replicated by direct addition of Dex to CM^{CTRL} indicates that the reduced migration is the result of changes in the CAF-derived secretome. In support, some of the components of CM that are suppressed by GC treatment can influence cell motility. uPA release from TGFβ-stimulated endometrial stromal cells increases migration of human microvascular ECs [408], whilst knock-down of the ANGPTL2 gene impairs migration of endothelial colony forming cells [409]. The same is true for HGF/SF and tenascin C, which we have previously shown to be downregulated by GCs [389]. HGF/SF has well-

known mitogenic and motogenic actions on ECs [410,411] and, thus, a reduction in HGF/SF could explain impaired HUVEC migration. Tenascin C promotes EC migration by binding to annexin II on the target cell surface, as well as by promoting phosphorylation of focal adhesion kinase [412,413]. Thus, decreased levels of these proteins in CM from Dex-treated CAFs are likely to explain the impaired motogenic response seen in HUVECs. Prostaglandins, especially PGE_2 and PGI_2, are known pro-angiogenic factors that directly induce EC survival, migration and tube-formation by activating respective receptors [406,414]. Therefore, insufficient levels of these factors in CM^{DEX} may have also negatively influenced HUVEC migration.

It was notable that, in contrast to the 2-dimensional assay with HUVECs and HAoECs (Figure 30), CM^{CTRL} did increase the number and length of vascular outgrowth formation in mouse aortic explants cultured *ex vivo* (Figure 32, Figure 33). This is unlikely to be simply due to a functional difference between umbilical vein and aortic ECs as single cultures of HUVECs and HAoECs responded in a similar way to CM in the TLS assay. Outgrowth formation in this assay is dependent on growth factor release from adventitial inflammatory cells [415]. Concomitant herewith, It is notable that the most dramatic change in transcript expression in HUVECs treated with CM was a 2-fold increase in IL-6 (Figure 31D), a pro-inflammatory cytokine that can influence angiogenesis. IL-6 and indeed many inflammatory proteins were not detected in the CAF-derived conditioned medium (Addendum 2, Supplementary Table 2). It has been reported that IL-6 stimulates angiogenesis directly leading to increased proliferation and migration of ECs [399], as well as endothelial progenitor cells [398]. This suggests the presence of inflammation-stimulating molecules in the CAF-derived secretome that are also sensitive to down-regulation by GCs. These results suggest that CM from CAFs increases angiogenesis indirectly by stimulation of growth factor release by other (non-endothelial) cells in the vascular wall. The reduced effect observed with CM^{DEX} can be attributed to both Dex-driven reduction of certain factors from CAF secretome and residual Dex in the medium, as addition of a comparable concentration of Dex to CM^{CTRL} had a similar effect, but slightly less pronounced (Figure 32). This is consistent with the well-documented direct angiostatic properties of GCs [344,386,387,397], including suppression of outgrowth formation in the aortic ring assay [397].

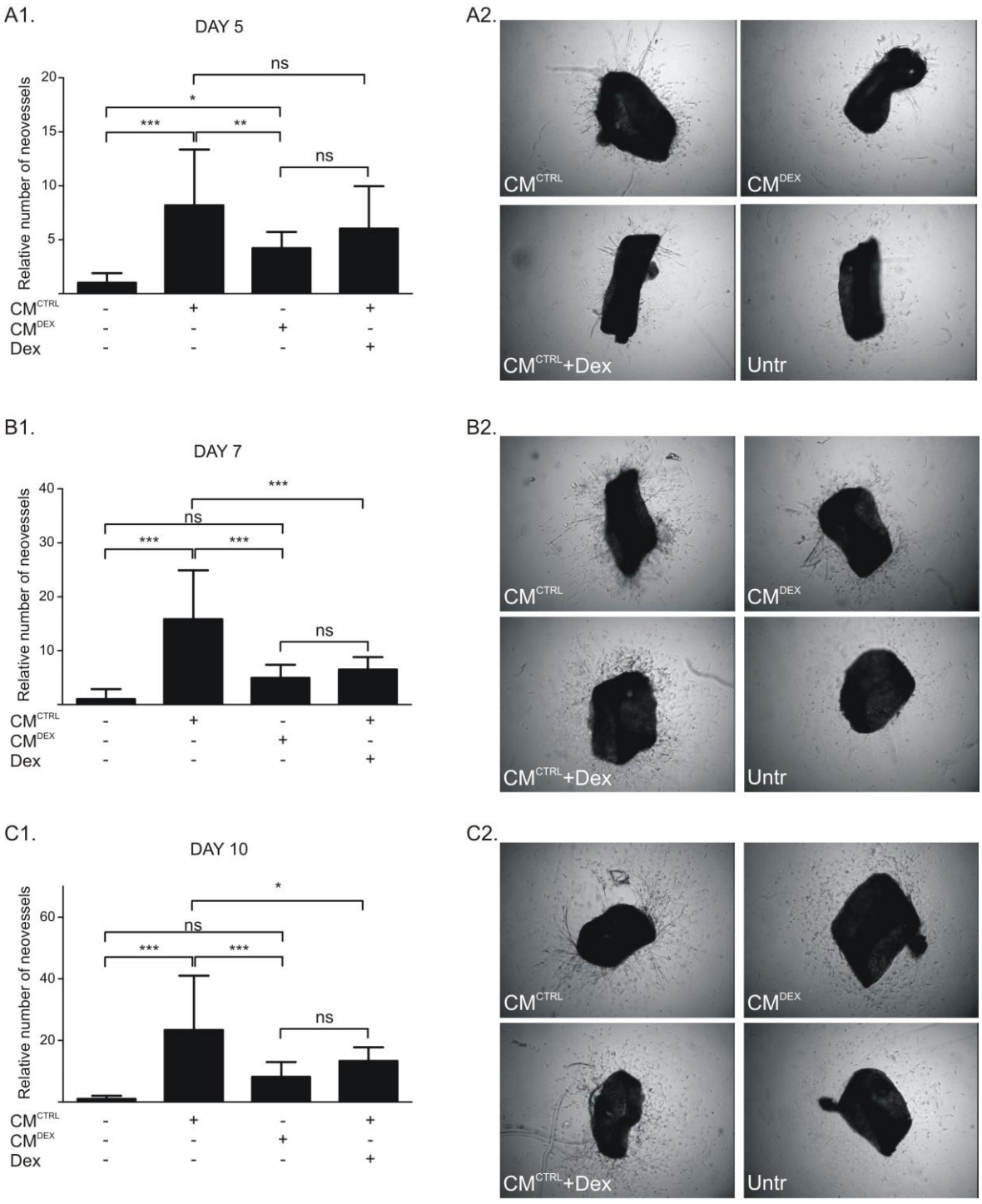

Figure 32. Conditioned medium from CAFs promotes neovessel formation in mouse aortic rings embedded in collagen. (A, B, C) Explants were prepared from aortas isolated from adult male C57BL/6 mice. After embedding in collagen, aortic rings were cultured in serum-free DMEM (control) or treated with CM^{CTRL}, CM^{DEX} or CM^{CTRL}+Dex (50nM), in 1:1 ratio with serum-free DMEM. Vascular sprouts were quantified after 5 days (A1, A2), 7 days (B1, B2) and 10 days (C1, C2) in culture. Left panel histograms represent the mean ± SD of six independent experiments. Results were analyzed using a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test. ns: not significant, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

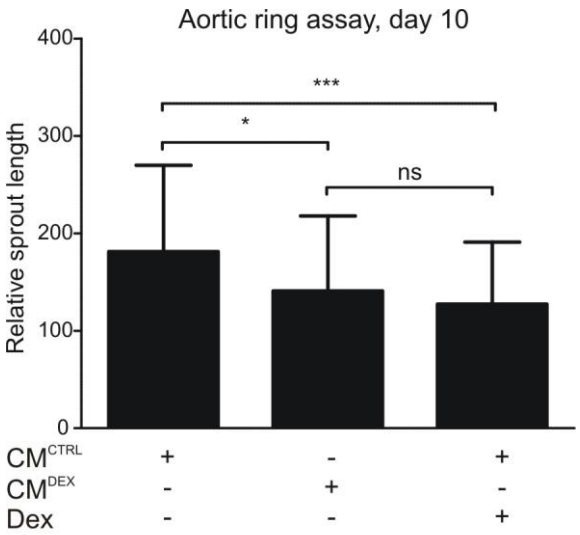

Figure 33. Conditioned medium from CAFs increases outgrowth length in mouse aortic rings embedded in collagen. Explants were prepared from aortas isolated from adult male C57BL/6 mice. After embedding in collagen, aortic rings were treated with CM^{CTRL}, CM^{DEX} or CM^{CTRL}+Dex (50nM), in a 1:1 ratio with serum-free DMEM. Images of explants and vascular sprouts were captured after 10 days and measurement of outgrowth length was performed using ImageJ software [362]. Results are the mean ± SD of six independent experiments and were analyzed using a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test. ns: not significant, *: $p<0.05$, ***: $p<0.001$.

In conclusion, this investigation has demonstrated that colon cancer-derived CAFs secrete pro-angiogenic factors and stimulate endothelial cell migration. This migration is inhibited by exposure of the CAFs to GCs which alter the components of the CAF secretome. A similar modulation of angiogenesis appears to be the result of indirect interaction of CM with non-endothelial vascular cells, possibly through activation of vascular inflammatory pathways. This work suggests that treatment with GCs may reduce the ability of CAFs to stimulate endothelial cell migration and angiogenesis, through both direct and indirect effects on the vascular wall.

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CHAPTER 3: ARTICLE 3.

Glucocorticoids decrease colon cancer cell proliferation and invasion via effects on cancer-associated fibroblasts

Zuzanna Drebert[1,2], Elly De Vlieghere[1,2], Jolien Bridelance[3], Olivier De Wever[1,2], Karolien De Bosscher[2,4], Marc Bracke[1,2], Ilse M. Beck[1,2,5]

[1] Laboratory of Experimental Cancer Research, Department of Radiation Oncology & Experimental Cancer Research, Ghent University, Ghent, Belgium

[2] Cancer Research Institute Ghent (CRIG), Ghent, Belgium

[3] Molecular Signaling and Cell Death Unit, Inflammation Research Center, VIB, Ghent, Belgium.

[4] Receptor Research Laboratories, Nuclear Receptor Lab, VIB Medical Biotechnology Center, Ghent University, Ghent, Belgium

[5] Department Health Sciences, Odisee University College, Ghent, Belgium;

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Abstract

Cancer-associated fibroblasts (CAFs) support cancer growth, invasion, and metastasis. Glucocorticoids (GCs), drugs often administered together with chemotherapy, are steroidal ligands of the glucocorticoid receptor (GR), a transcription factor which upon activation regulates expression of multiple genes involved in suppression of inflammation. We have previously shown that in dexamethasone (Dex)-treated CAFs derived from colon cancer, production and secretion of several factors related to cancer progression, such as tenascin C (TNC) and hepatocyte growth factor (HGF), were strongly suppressed.

In this study we show that GCs can neutralize the cancer cell-promoting properties of CAFs. Conditioned medium from solvent-treated CAFs (CM^{CTRL}) stimulates proliferation, motility and invasive morphotype of GR-deficient HCT8/E11 colon cancer cells. Yet, HCT8/E11 proliferation and invasive morphotype are impaired upon treatment with conditioned medium from Dex-treated CAFs (CM^{DEX}), but HCT8/E11 cell migration is slightly increased under these conditions. Moreover, expression and potential activity of MMP-2 is also reduced in CM^{DEX} compared with CM^{CTRL}. These combined *in vitro* results concur with the results from *in vivo* chick chorioallantoic membrane assays, where the co-cultures of CAFs with colon cancer cells displayed impaired tumor formation and cancer cell invasion due to Dex administration. Combined, GC treatment influences cancer cell behavior indirectly through effects on CAFs.

3.1. Introduction

Colorectal cancer (CRC) is one of the most common malignant neoplastic diseases in Europe and Northern America [4]. CRC's morbidity is linked to western dietary lifestyle, age, obesity, smoking, alcohol consumption, lack of physical activity, and certain hereditary diseases [416]. Despite an improvement in treatment, CRC accounted for nearly 10% of cancer-related deaths in 2012 [4].

Cancer development is driven by sustained proliferative signaling, resistance to apoptosis and to growth suppressors, angiogenesis, escape from immune response, reprogramming of metabolism, invasion, and metastasis [8]. Last decades' progress in cancer research was enhanced by an improved understanding of the importance of the tumor microenvironment. Stromal components including inflammatory cells, cells forming tumor vasculature and lymphatics, myofibroblasts, and the extracellular matrix are not passive bystanders. On the contrary, they play a crucial role in virtually every step of cancer progression. Researching this complex net of interactions between certain components of the tumor microenvironment creates opportunities for diagnosis and therapy [8,68].

Myofibroblasts present in the tumor stroma are also known as cancer-associated fibroblasts (CAFs). They mostly differentiate from resident fibroblasts and share attributes of smooth muscle cells and fibroblasts and express markers, such as α-smooth muscle actin, fibroblast activation protein-α and vimentin [417]. CAFs are recruited by cancer cells at the invasion front of the tumor and they influence cancer cells via cell-to-cell contact or via secreted products, such as cytokines, chemokines, enzymes, and other factors [76,347]. CAFs are abundantly present in CRC compared to normal mucosa, both at primary and metastatic sites, which is related to poor overall and relapse-free survival [167]. CAFs were shown to contribute to the following tumor-promoting actions: cancer proliferation, induction of angiogenesis, protection from anti-tumor immune responses, activation of invasion, and promotion of metastasis [127].

Depending on the type and stage of the cancer different types of treatment are implemented. Most often patients receive a combination of therapies, which include surgery, radiation therapy, chemotherapy, immunotherapy, targeted therapy, hormone therapy, and stem cell transplant [5]. Cancer treatment, however, faces problems of therapy resistance, which can be also triggered by cancer stroma. Environment-mediated drug resistance (EMDR), whereby CAFs protect cancer cells by secreting a multitude of cytokines is linked with cancer aggressiveness and poor response to treatment [142].

Glucocorticoids (GCs) are drugs that are often used in combination with chemotherapy, hormonal therapy, radiotherapy, and surgery of solid tumors, in order to relieve symptoms of the disease and the associated side-effects of these treatments [278]. GCs are steroidal compounds, essential

in regulating metabolism, blood pressure, reaction to stress, and immune response [231]. GCs are able to bind and activate the glucocorticoid receptor (GR). Ligand-bound GR is translocated from the cytoplasm to the nucleus, where it acts as a homodimerized transcription factor to positively regulate expression of numerous specific target genes by binding to glucocorticoid responsive elements (GREs). Furthermore, GR in its monomeric form can tether other transcripion factors, such as NFκB or AP-1, resulting in inhibition of transcription of many pro-inflammatory genes. These two major mechanisms are known respectively as transactivation and transrepression [188,220]. GR actions result in suppression of inflammation and therefore GCs are widely used in the clinic against many inflammatory disorders, such as asthma, allergies, and autoimmune diseases [204,231]. Besides their anti-inflammatory properties, GCs also serve as angiostatic agents in infantile hemangiomas [276] and form a treatment of hematological malignancies, such as multiple myeloma and lymphoma [279]. The role of GR modulation in solid tumor biology, however, is still not fully understood. This is also a topic of controversy, since the result of GC treatment depends on the primary site of the tumor and extends from possible detrimental effects in lung cancer, over neutral in gastrointestinal cancer to positive effects in prostate cancer [278,281]. Interestingly, GR mRNA levels are elevated in the stroma of breast cancer, compared to the healthy breast tissue. Moreover, in breast cancer, there is a positive correlation between GR mRNA levels in the tumor stroma and the tumor stage [418]. Lastly, approximately 50% of human colon tumors are GR-positive and the increased GR expression in colorectal adenocarcinoma patients is actually linked with a poor prognosis [312,419].

In our previous studies, we have shown that GR modulation has an impact on the colon cancer-derived CAFs biology and function. Treatment with the GC dexamethasone (Dex) diminished inflammatory gene expression, and moreover, generated changes in the CAF secretome, including suppression of expression of hepatocyte growth factor/scatter factor (HGF/SF) and tenascin C (TNC) [389,420]. HGF/SF is a well-documented factor with mitogenic and motogenic properties on epithelial and endothelial cells, that acts via the c-Met receptor [134]. TNC is an extracellular matrix protein abundant during the wound healing process and also involved in cancer invasion via low-affinity binding to the epidermal growth factor receptor (EGFR) [381]. CAF-derived HGF and TNC were proven to be both necessary - but not sufficient on their own - to promote colon cancer cell invasion *in vitro*, via RhoA and Rac pathways [77]. Interestingly, both HGF and TNC were strongly downregulated in CAFs, at mRNA and protein levels, following a GC treatment. Therefore, we wanted to establish the relevance of these GC-driven changes in CAF secretomes on cancer cell proliferation, migration, and invasion, and as such, to provide a novel insight into the role of GCs in the colon cancer microenvironment.

3.2. Materials & Methods

3.2.1. Cells and reagents

HCT8/E11 human colorectal adenocarcinoma (ATCC number: CCL-244) [421], in-house engineered HCT8/E11-luc cells [422], HCT116 human colon carcinoma (ATCC number: CCL-247) [423] and CT5.3hTERT human stromal colon cancer-derived CAFs [77,171] were cultured in DMEM (Life Technologies, Merelbeke, Belgium) supplemented with 10% fetal calf serum (Greiner bio-one, Wemmel, Belgium), 100 U/ml penicillin and 0,1 mg/ml streptomycin (Life Technologies) at 37°C with 10% CO_2. DMEM used in experiments was serum-free or supplemented with charcoal-stripped serum (Life Technologies). The GCs dexamethasone (Dex), hydrocortisone (Hcrt), prednisolone (Pred) and fluocinolone acetonide (FA) were purchased from Sigma-Aldrich (Diegem, Belgium) and dissolved in ethanol. A selective GR modulator (SEGRM) compound A (CpdA) was prepared according to De Bosscher, *et al.* [268]. Recombinant murine tumor necrosis factor (TNF)α was prepared as described by Vanden Berghe, *et al.* [355] and dissolved in serum-free DMEM. Firefly D-luciferine was purchased from PerkinElmer (Zaventem, Belgium) and prepared according to the manufacturer's instructions. Human recombinant hepatocyte growth factor (hrHGF) was purchased from PromoKine (Heidelberg, Germany, cat no: c-64532), human recombinant tenascin C (hrTNC) was purchased from R&D Systems (Abingdon, UK, cat no: 3358-TC-50) and both were resuspended in PBS.

Table 7. List of compounds used to incubate CT5.3hTERT CAFs and subsequent preparation of conditioned media. The concomitant ethanol concentrations are identical in all conditions.

Compound	Concentration	Abbreviation used for the CM
Ethanol	0.1%	CM^{CTRL}
Dexamethasone	1 µM	CM^{DEX}
Hydrocortisone	1 µM	CM^{HCRT}
Prednisolone	1 µM	CM^{PRED}
Fluocinolone acetonide	1 µM	CM^{FA}
Compound A	10 µM	CM^{CPDA}

3.2.2. Conditioned medium preparation

Conditioned medium (CM) was prepared according to previous protocols [77]. Briefly, supernatants were collected from $10x10^6$ CT5.3hTERT CAFs, which were cultured for 48h in serum-free DMEM and treated with solvent (ethanol), Dex or, optionally, with Hcrt, Pred, FA or a SEGRM CpdA in concentrations listed in Table 7. Subsequently, CM was 10-fold concentrated with

centrifugal filter tubes with a 3kDa cut-off (Amicon Ultra, Merck Millipore, Darmstadt, Germany) and filter-sterilized prior to storage at -20°C.

3.2.3. Cell lysis and western blot analyses

Cells were collected from HCT8/E11, HCT116 and/or CT5.3hTERT cultures and subsequently washed with PBS. Protein lysates were made using TOTEX buffer (20mM Hepes/KOH pH 7.9; 0.35M NaCl; 20% glycerol; 1% NP40; 1mM $MgCl_2$; 0.5mM EDTA; 0.1mM EGTA; 2mM pefabloc; 10µg/ml aprotinin). Protein concentration was determined via the Lowry method [392]. Alternatively, 10-fold concentrated conditioned medium from CAFs treated with solvent or Dex (1µM) for 48h, was prepared for western blot analysis using SDS sample buffer (50mM Tris pH6.8; 2% SDS; 10% glycerol; bromophenol blue, 100mM DTT).

Samples were subjected to SDS-PAGE followed by a standard Western Blot protocol, as described by Santa Cruz (Santa Cruz Biotechnology, CA, USA). As primary antibodies, we used anti-GR (H-300) (1/1000, Santa Cruz Biotechnology, cat no: sc-8992), anti-tubulin (1/4000, Sigma-Aldrich, cat no: T5168) and anti-human MMP-2 (1/500, R&D Systems, cat no: AF902). We used species-specific HRP-linked secondary antibodies anti-mouse, anti-rabbit (GE Healthcare, Diegem, Belgium, cat no: NA931V, NA934V) and anti-goat (Santa Cruz Biotechnology, cat no: sc-2020). For visualization of the results we used ECL solution (Thermo Scientific, Gent, Belgium) and X-Ray films (GE Healthcare) or alternatively WesternBright Quantum HRP substrate (Advansta, CA, USA) and a ProXima imaging platform 2850 with ProXima AQ-4 software (Isogen Life Science, De Meern, The Netherlands). Quantification of western blot results was performed using ImageJ software [362] according to previous protocols [389].

3.2.4. RNA isolation and RT-qPCR

HCT8/E11, HCT116 and CT5.3hTERT cells were collected and total RNA was isolated. Alternatively, HCT8/E11 cells were first induced with solvent, Dex (1µM) or a SEGRM CpdA (10µM) for 1h and then co-treated with TNFα or equivalent volume of DMEM for another 5h, before total RNA was isolated. We used TRIzol reagent (Life Technologies) to isolate the total RNA from these cells, which was subsequently followed by reverse transcription (RT), performed with an iScript kit (Bio-Rad), and quantitative PCR (qPCR) using Lightcycler 480 SYBRGreen I Master reagents (Roche Diagnostics, Rotkreuz, Switzerland), all according to the manufacturers' instructions. We performed qPCR reactions in triplicates using the Lightcycler® 480 system (Roche Diagnostics) and the following protocol: A) initial denaturation 95°C, 5'; B) 40 cycles of denaturation 95°C, 15'', annealing and elongation 60°C, 45". Primer sequences are available in Addendum 5 (Supplementary Table 4). Further, results were normalized to the results obtained for the respective geometric mean of 3 housekeeping genes (GAPDH, PPIB, 36B4). Final results are

displayed as relative mRNA expression, in which the solvent control condition was set as 1 and all other conditions were recalculated accordingly.

3.2.5. Gelatin zymography

Conditioned medium from CT5.3hTERT cells treated with solvent or Dex (1µM) for 48h was 10-fold concentrated and applied to the zymography protocol as described [424]. Briefly, conditioned medium samples were subjected to SDS-PAGE using 10% polyacrylamid-0.1% gelatin gels. Next, gels were incubated in renaturing solution (2.5% Triton-X) for 30 min, then washed twice with dH2O and incubated at 37°C in a developing buffer (50mM Tris-HCl pH 7.8, 0.2 M NaCl, 5mM $CaCl_2$) overnight. Subsequently, gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad, Temse, Belgium) for 1h and then destained with destaining solution (10% methanol, 5% acetic acid). Quantification of zymogram results was performed with ImageJ software as explained earlier [362]. As this particular assay may not represent the actual state of MMPs released by cells, due to possible inactivation via noncovalent binding with tissue inhibitors of MMPs (TIMPs), these results are expressed as “potential enzyme activity”.

3.2.6. Cell proliferation assays

To assess proliferation of HCT8/E11-luc cells we performed a co-culture assay and assays using CM from CAFs, based on previous protocols [422]. In a co-culture assay CT5.3hTERT cells were seeded in 24-well plates together with HCT8/E11-luc cells at a 10:1 ratio and subjected to solvent or Dex (1µM) incubation. After a 72h incubation, D-luciferine (150 µg/mL) was added to the wells and luciferase activity was measured with the *In vivo* Imaging System Lumina II (IVIS®, Caliper Life Science, Hopkinton, MA, USA). Similarly, HCT8/E11-luc cells were seeded in 24-well plates (10^4/well) and after 24h cells were treated with DMEM, CM^{CTRL} or CM^{DEX}. D-luciferine (150 µg/mL) was added to the wells 72h post treatment and bioluminescence was measured using the IVIS. Results were analyzed via Living Image® software (Caliper Life Science).

Additionally, we performed a sulforhodamine-B (SRB) test, as described previously [394] using the parental cell line HCT8/E11. Briefly, cells were seeded in 96-well plates ($5x10^3$/well) and treated with DMEM, CM^{CTRL} or CM^{DEX} for selected time points (24h, 48h and 72h). Following fixing, staining and washing steps, plates were scanned using a ParadigmTM Detection Platform (Beckman Coulter®, Krefeld, Germany) with SoftMax® Pro 6.1 software. Results are expressed in a scale, where the untreated post treatment condition at 72h was set at 1 and all other conditions were recalculated accordingly.

3.2.7. Cell morphology assay

The cell morphology assays on collagen were performed as described by De Wever, *et al.* [425]. Briefly, single cell suspensions of $7x10^4$ HCT8/E11 were seeded in 6-well plates, or alternatively, $1.2x10^4$ cells were seeded in 24-well plates, all on a layer of type I collagen (derived from rat tail; 1mg/ml; Santa Cruz Biotechnology). Cells were treated with DMEM or with CM from CAFs as indicated in the figure legends. Cell morphology was observed 24h post treatment under a phase-contrast microscope (Leica DMI3000B with LAS4.1 software) and digital images from 10-15 microscopic fields (20 x magnifications) were taken for further evaluation. Cells with spread and round morphology were counted and the results per microscopic field are expressed as a relative cell spread index.

3.2.8. Migration assay

HCT8/E11 cell migration was assessed using a Transwell system. Cells were seeded in serum-free DMEM on Transwell inserts ($5x10^4$ cells/insert) with 8.0µm pores (Corning Inc., Lasne, Belgium, cat no: 3422) and left to migrate through the membrane for 24h towards serum-free DMEM, CM^{CTRL} or CM^{DEX}, which was applied in the lower compartments of the Transwell system. After 24h inserts were removed and the inside parts of these inserts were gently wiped with cotton swabs to remove cells which did not migrate. Next, the membranes were fixed with ice-cold methanol and washed 3 times for 5 min in PBS. Membranes were then stained with DAPI (0.4µg/ml), washed with PBS and subsequently mounted on microscope glasses. Membranes were observed under a fluorescence microscope (Zeiss Axiovert 200M, Carl Zeiss, Micro-Imaging, Heidelberg, Germany), which enabled counting the cells that migrated through the porous membrane. Cells were counted per microscopic field (10 x magnification), and 10 fields per condition were assessed.

3.2.9. Adhesion assays

Cell-to-cell adhesion assays between CAFs and HCT8/E11 cells were performed as described [422]. CT5.3hTERT CAFs were cultured in 24-well plates until confluency. Subsequently, cells were incubated with solvent or Dex (1 µM) for 24h prior to an additional seeding of 10^4 HCT8/E11-luc cells/well. After 24h of co-culturing, cells were washed twice with DMEM in order to remove the non-adherent cells. Subsequently D-luciferine (150 µg/mL) was added to the wells and luciferase activity was measured using the IVIS system.

HCT8/E11 cancer cells' adhesion to collagen coating was measured as described [78]. Briefly, HCT8/E11 cells (10^4/well) were seeded in quadruplicates in type I collagen-coated (50 µg/ml) E-16 plates (ACEA Biosciences, Sand Diego, CA, USA). Cells were seeded in serum-free DMEM, CM^{CTRL} and CM^{DEX}. Cell-electrode impedance indicating cell adhesion was assessed every 5 minutes for

24h using xCELLigence RTCA SP (ACEA Biosciences). Cell adhesion is reported as a relative cell index and areas under the curve (AUC) were calculated for the first 60 minutes of each treatment.

3.2.10. Chorioallantoic membrane (CAM) assay

The chick embryo CAM assay was performed according to [426] and slightly adjusted. Briefly, fertilized eggs from a local hatchery were incubated at 37.8°C and 50% humidity in a poultry egg incubator (R-COM 50 Digital Egg Incubator, Gyeonggi-do, South Korea). At day 3 of embryonic development, 2-3ml of albumen was removed with a sterile needle in order to lower the level of the CAM. Additionally, an opening of approximately $1cm^2$ was made in the eggshell in order to evaluate the embryos' state and eliminate dead or non-fertilized eggs. The window was then covered with a semipermeable polyurethane film (Suprasorb F, Lohmann & Rauscher, Neuwied, Germany). At day 9, single cell suspensions of 10^6 HCT8/E11 cancer cells together with $2.5x10^6$ CT5.3hTERT CAFs were seeded onto the CAM in Matrigel™ drops (100µl/CAM). Cells were treated while seeding with solvent or Dex (1µM) and re-treated 48h later in 20µl Matrigel drops. Five days after seeding, tumors were observed under the stereomicroscope (Leica Microsystems, Diegem, Belgium) and digital images were taken. CAM fragments containing tumors were harvested and fixed in buffered formaldehyde (4% formaldehyde, 4g/L $Na_2PO_4 \cdot H_2O$, 6.5g/L Na_2HPO_4). Subsequently, these samples were embedded in paraffin, sectioned and subjected to hematoxylin-eosin staining, as described by Sigma-Aldrich. These prepared slides were evaluated for tumor shape (sphericity) and cancer cell infiltration into the CAM's mesenchymal layer, on a scale from 1 to 5 (Addendum 3, Supplementary Table 3).

3.2.11. Statistical analyses

We performed statistical analyses using GraphPad Prism 5.03 with the unpaired student t-test, Mann-Whitney test or one-way analysis of variance (ANOVA) with Tukey's multiple comparisons post-test, where applicable as indicated in the figure legends. A p-value of $p < 0.05$ was considered statistically significant.

3.3. Results

3.3.1. HCT8/E11 cells do not express a functional GR

In order to investigate the role of glucocorticoid receptor (GR) modulation in CAFs and its subsequent effects on colon cancer cells, we chose a colon cancer cell line that does not express a functional GR, enabling us to research the direct influence of glucocorticoid (GC) treatment limited solely to CAFs. Western blot and qPCR analyses revealed that HCT8/E11 colon cancer cells display lack of GR at both mRNA and protein levels (Figure 34A, B). Another colon cancer cell line, HCT116, showed a moderate expression of the receptor, both at mRNA and protein levels, as

compared to colon cancer-derived CT5.3hTERT CAFs, which express relatively high levels of both GR mRNA and GR protein (Figure 34A, B). Corresponding with its GR-deficient status, the administration of the glucocorticoid Dex (1μM, 6h) to HCT8/E11 cells did not lead to a statistically significant upregulation of glucocorticoid-inducible leucine zipper (GILZ), a gene known to be highly expressed following GC treatment [234] (Figure 34C). In HCT116 cells, GILZ was 5.5-fold upregulated due to Dex treatment while in CT5.3hTERT cells we observed an average 70-fold GILZ mRNA upregulation. Additional to the lack of GR transactivation in HCT8/E11 cells, these cells also did not display GR-mediated transrepression properties (Addendum 3, Supplementary Figure 10A, B). The treatment with TNFα led to an upregulation of NFκB-driven pro-inflammatory molecules, namely ICAM and MCP-1 in HCT8/E11 cells. However, a co-treatment with Dex did not lead to a suppression of expression of these molecules, in contrast to CT5.3hTERT cells, where this suppression was well pronounced (Figure 21) . The selective GR modulator (SEGRM) compound A (CpdA), a non-steroidal plant-derived molecule, yet able to modulate GR favoring its transrepressive actions [268,389], displayed a similar pattern as Dex. These combined results point to a lack of both GR transactivation and GR transrepression activities in the GR-deficient HCT8/E11 cells.

3.3.2. Secretion of MMP-2 by CAFs is affected by Dex-treatment.

MMP-2 belongs to the family of matrix metalloproteinases and has been studied as one of the biomarkers of colorectal cancer [133]. In order to investigate MMP-2's presence in CAF secretomes, we performed western blot analyses, which showed that MMP-2 levels are decreased in the conditioned medium of these cells following 48h Dex exposure (Figure 35A, B). An MMP-2 activity assay, gelatin zymography, revealed that the majority of MMP-2 was secreted in an inactive form (pro-enzyme), as pro-MMP-2 (Figure 35C). The potential activity of the MMP-2 pro-enzyme and of the MMP-2 active form decreased in samples obtained from Dex-treated cells, CM^{DEX}, compared to CM^{CTRL} (Figure 35C, D), which is in line with the protein expression status. However, only the difference in pro-enzyme potential activity obtained statistical significance. Although MMP-9 could not be visualized via Western blot analyses (data not shown), pro-MMP-9 could be visualized via zymography at very low signal compared to MMP-2. Pro-MMP-9's potential activity was also decreased in CM^{DEX} (Figure 35E).

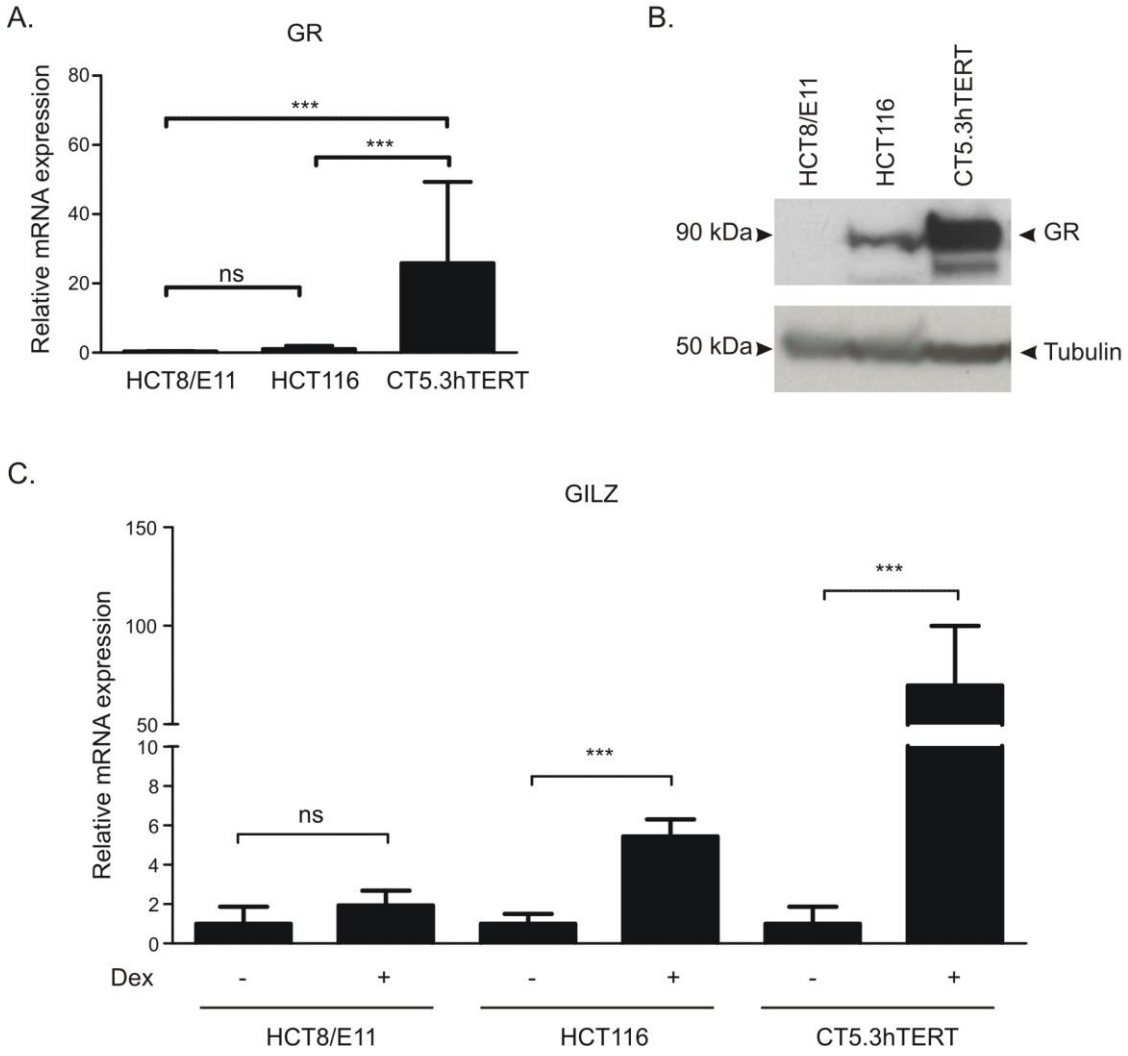


Figure 34. HCT8/E11 colon adenocarcinoma cells do not express a functional glucocorticoid receptor. (A) mRNA isolated from HCT8/E11, HCT116, and CT5.3hTERT cells was subjected to RT-qPCR assaying GR mRNA levels. Results were normalized to the respective geometric mean of GAPDH, PPIB, and 36B4 reference genes' mRNA levels. Results are shown as the mean ± SD of three independent experiments and statistical analysis was performed using a one-way ANOVA and Tukey's multiple comparisons post-test. ns: not significant, ***: $p < 0.001$. (B) Total cell lysates obtained from HCT8/E11, HCT116, and CT5.3hTERT cells were subjected to Western Blot analysis for the detection of GR and the loading control tubulin. Results are representative of at least three independent experiments. (C) HCT8/E11, HCT116, and CT5.3hTERT cells were treated with solvent or Dex (1µM) for 6h. Isolated total mRNA was subjected to RT-qPCR assaying GILZ mRNA levels. Results were normalized to the respective geometric mean of GAPDH, PPIB, and 36B4 reference genes' mRNA levels. Results are shown as the mean ± SD of three independent experiments and statistical analysis was performed for pairwise comparisons using an unpaired t-test. ns: not significant, ***: $p < 0.001$.

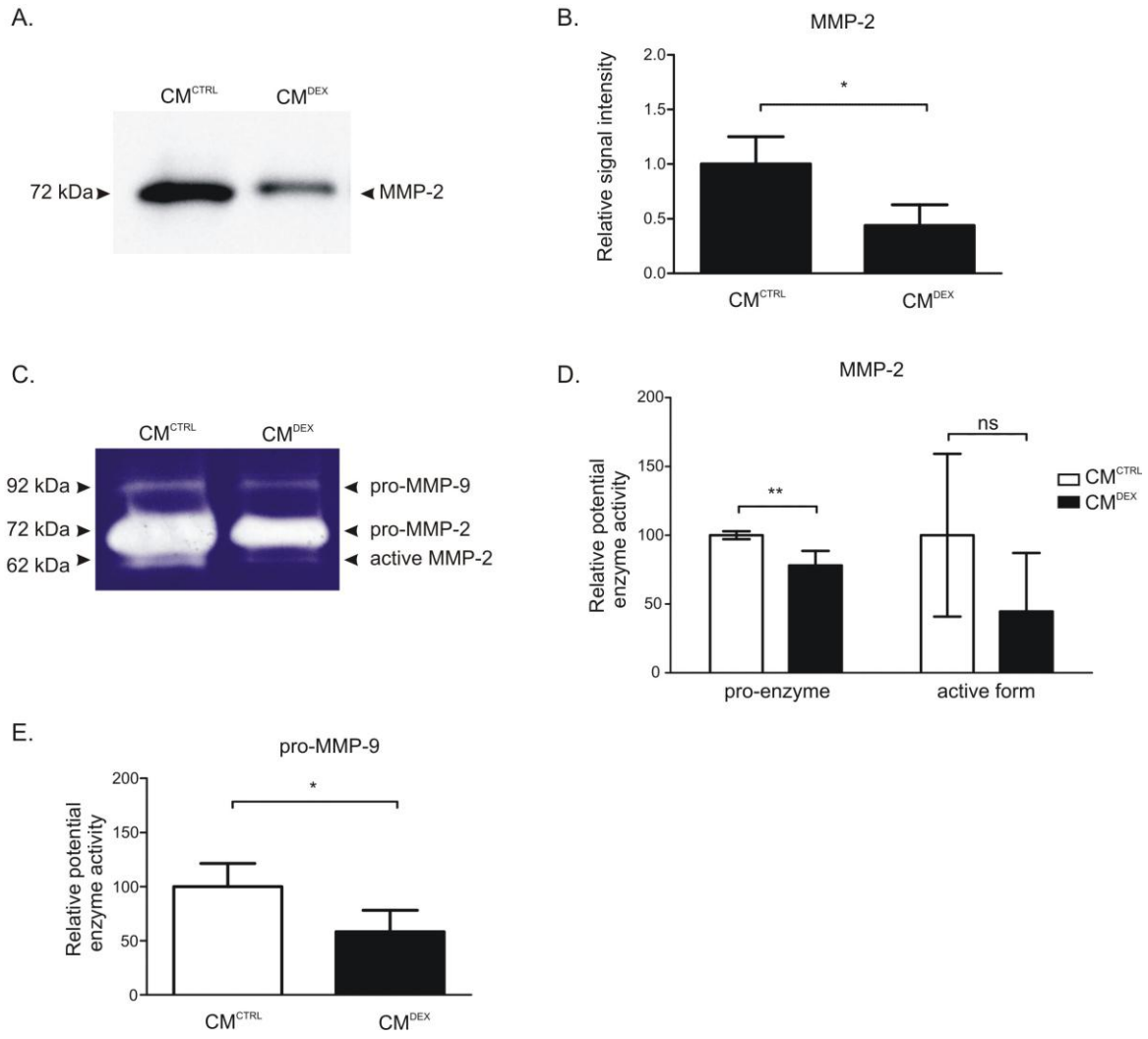


Figure 35. MMP-2 expression and potential activity is decreased in the conditioned medium from Dex-treated CAFs (A, B) CT5.3hTERT cells were treated with solvent or Dex (1µM) in serum-free DMEM. After 48h cell supernatants were collected and 10-fold concentrated. Such prepared conditioned medium samples (CM^{CTRL} and CM^{DEX}) were subjected to (A, B) western blot analysis for the detection of MMP-2 and (C, D, E) gelatin zymography for detection of MMP-2 and MMP-9 potential activity. Signal quantification was performed using ImageJ software. Images (A, C) are representative of 4 independent experiments. Results (B, D, E) are shown as the mean ± SD of four independent experiments and statistical analysis was performed using an unpaired t-test. ns: not significant, **: $p < 0.01$, *: $p < 0.05$.

3.3.3. Indirect impact of Dex treatment on HCT8/E11 through co-culture with CAFs affects proliferation but not adhesion of HCT8/E11 cells

To investigate whether Dex treatment could have a CAF-mediated effect on HCT8/E11 we performed a cell proliferation and cell-to-cell adhesion assay in a co-culture system. In the proliferation assay (Figure 36A), HCT8/E11-luc cells cultured together with CT5.3hTERT CAFs in a 1:10 ratio, displayed growth inhibition in the presence of Dex (1 µM, 72h) compared with solvent control-treated cells. An adhesion assay using a CAF confluent culture showed that pre-treatment of these cells with Dex (1µM, 24h) did not affect HCT8/E11-luc adhesion to CAFs and their secreted matrix (Figure 36B) in comparison to solvent control-treated cells.

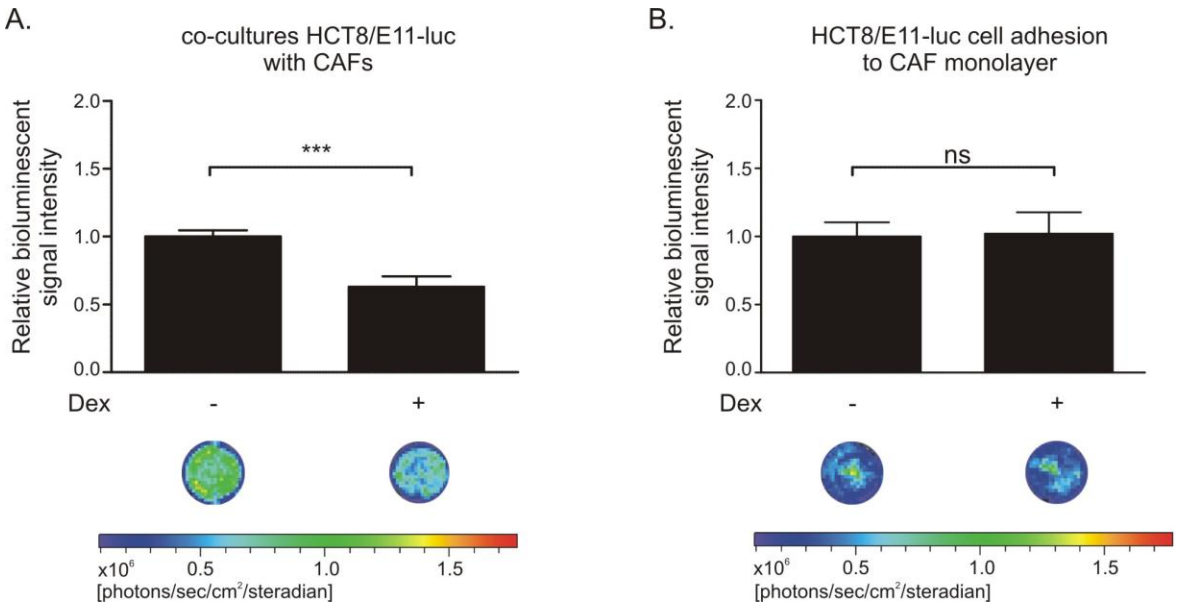


Figure 36. In the co-culture system, Dex treatment indirectly affects HCT8/E11 growth, but not cell adhesion to a monolayer of CAFs. (A) HCT8/E11-luc cells were seeded together with CT5.3hTERT CAFs in a 1:10 ratio and treated with solvent or Dex (1µM) for 72h. Signal quantification was performed via bioluminescent imaging (IVIS). (B) CT5.3hTERT cells were treated with solvent or Dex (1µM). After 24h HCT8/E11-luc cells were seeded on top of the CAF monolayer and 24h later signal quantification of HCT8/E11-luc cells was performed via bioluminescent imaging (IVIS). (A, B) Images are visualizations of representative wells of each condition displayed as a bioluminescence activity heat-map. Solvent conditions were set at 1 and the Dex condition was recalculated accordingly. Results are shown as the mean ± SD of three independent experiments and statistical analysis was performed using an unpaired t-test. ns: not significant, ***: $p < 0.001$.

3.3.4. HCT8/E11 cell proliferation, morphology, and motility are changed due to exposure to CM^{DEX} compared to CM^{CTRL}.

To assess whether the growth-inhibitory effects of Dex-treated CAFs originate from changes in the CAF secretome, we performed experiments using CAF-derived conditioned medium (CM^{CTRL}) and CM from Dex-treated CAF (CM^{DEX}). Via cell viability and metabolic activity assays (MTT), we observed that neither CM^{CTRL} nor CM^{DEX} impaired cell survival tested in a confluent culture of HCT8/E11 after 72h of treatment (Addendum 3, Supplementary Figure 11). In a cell proliferation experiment with HCT8/E11-luc cells (Figure 37A), we observed that both CM^{CTRL} and CM^{DEX} promoted colon cancer cell growth, compared to the control treatment with DMEM. However, CM^{DEX} had a significantly weaker impact than CM^{CTRL} on HCT8/E11-luc growth after 72h of incubation. These results are consistent with data obtained from an SRB assay in which proliferation of the parental HCT8/E11 cell line was assessed in the presence of CM from CAFs (Figure 37B). Also in this situation, 72h incubation with CM promoted cell growth compared to DMEM, and effects of CM^{DEX} were less pronounced than those of CM^{CTRL}.

Changes in cell morphology into a stretched, elongated shape accompany epithelial-to-mesenchymal transition (EMT) and a subsequent cell invasion [8]. In an *in vitro* cell morphology

assay on collagen, HCT8/E11 cells treated with CM^{CTRL} adopted a spread morphotype, characteristic for invasive cells (Figure 37C, Addendum 3, Supplementary Figure 12). Treatment with CM^{DEX} resulted in a significantly diminished number of cells with such invasive morphotype. Moreover, cell morphology effects obtained with CM^{DEX} were also observed with CM from CAFs treated with other GCs, namely FA, Pred and Hcrt (Addendum 3, Supplementary Figure 13A). Furthermore, although unlikely due to the GR-defective status of HCT8/E11 cells, we could rule out direct effects of residual GC in the CAF-derived CM. The addition of Dex to CM^{CTRL}, in order to mimic the direct potential impact of residual Dex in CM^{DEX}, as expected did not affect the pro-invasive influence of CM^{CTRL} (Addendum 3, Supplementary Figure 13B), showing that the effects of CM^{DEX} occur indeed due to changes in the CAF secretome and not due to residual GC.

As the selective GR modulator compound A (CpdA), is not able to trigger GR transactivation, we used this GR modulator in the morphotype assays to assess whether GR transactivation or GR transrepression events could lie at the basis of the effect of CAF-derived CM on HCT8/E11 morphotype changes. When applying CM derived from CpdA-treated CAFs (CM^{CPDA}) no difference compared to CM^{CTRL} could be observed, suggesting indeed GR-mediated transactivation mechanisms as the basis of changes in CAF-derived CM (Addendum 3, Supplementary Figure 13C). Nevertheless, GR-mediated non-genomic events cannot be excluded at this time.

A GC-driven inhibition of HGF and TNC expression in CAFs occurring most likely via GR transactivation events was reported earlier [389,420]. We assessed whether these changes could be the main cause of the affected HCT8/E11 cell morphotype changes. However, HCT8/E11 cells seeded on collagen and incubated with CM^{DEX} supplemented with either HGF (50 ng/ml) or TNC (2µg/ml) did not display an increased invasive morphotype above the levels obtained by the treatment with CM^{DEX} alone (Addendum 3, Supplementary Figure 14A). Combination of both HGF and TNC added to CM^{DEX} also did not result in a significant restoration of the invasive properties of CM above the CM^{DEX} level. However, a combined treatment with HGF and TNC did stimulate cell invasion when cells were incubated in DMEM, confirming their functionality (Addendum 3, Supplementary Figure 14B).

Increased cell motility facilitates cancer invasion [8] and it has been well-documented that CAFs promote cancer cell migration via secreted factors [347]. In a migration assay using porous membrane inserts (Transwell), we observed that the presence of CM^{CTRL} below the insert favored HCT8/E11 cell migration through the membrane, compared with a DMEM control (Figure 37D). CM^{DEX}, however, induced colon cancer cell migration slightly stronger compared to CM^{CTRL}.

Similarly to the co-culture experiments results, CAF-derived CM did not affect HCT8/E11 cell adhesion to a type I collagen coating (Figure 37E), which was measured via cell impedance (xCELLigence). Analysis of the area under the curve (AUC; Figure 37F) indicated that HCT8/E11

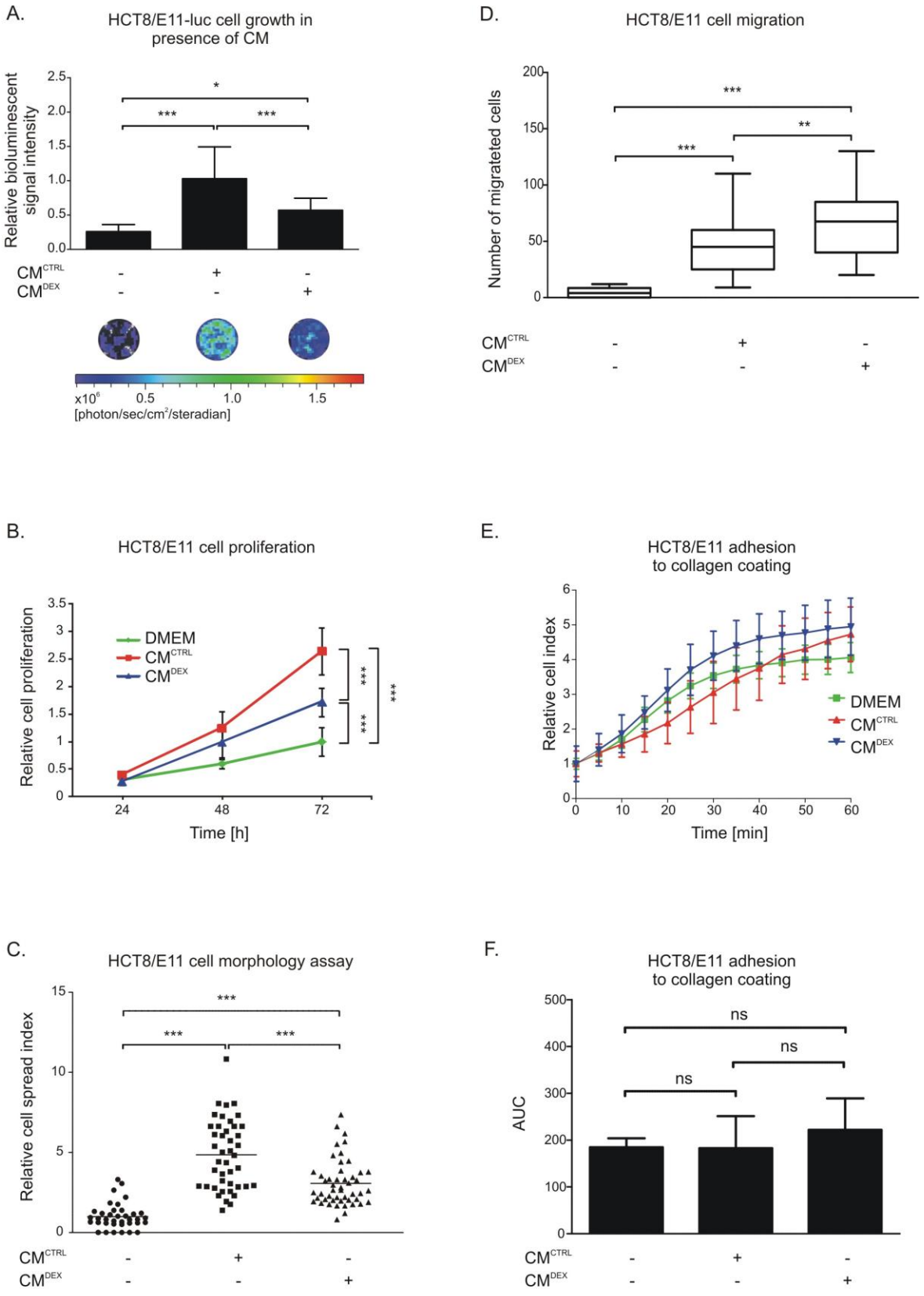
cells adhered to the collagen coating evenly, disregarding the treatment with CAF-derived CM^{CTRL} or CM^{DEX}.

In conclusion, CM from Dex-treated CAFs displayed diminished pro-invasive and pro-growth potential, but had stronger pro-migratory properties on HCT8/E11 colon cancer cells, as compared to CM from the control CAFs.

3.3.5. Dex treatment inhibits tumor formation *in vivo*.

The *in vivo* chorioallantoic membrane (CAM) assay is widely used as a model for tumor development, invasion, and angiogenesis [427]. HCT8/E11 cells seeded together with CT5.3hTERT CAFs in a drop of Matrigel were able to form tumors (Figure 38A). Application of Dex (1µM) for 5 days affected tumor shape, resulting in less spherical tumors (Figure 38B). Moreover, in Dex-treated tumors a significant inhibition of cancer cell infiltration into CAM's mesenchymal layer was observed (Figure 38C).

Figure 37. (next page) CM^{DEX} has a diminished potential to stimulate HCT8/E11 cell growth and invasive morphotype but can increase cell motility, as compared to CM^{CTRL}. (A) HCT8/E11-luc cells were cultured with serum-free DMEM, CM^{CTRL} or CM^{DEX} for 72h and signal quantification was performed by bioluminescent imaging (IVIS). Images are visualizations of representative wells of each condition displayed as a bioluminescence activity heat-map. The DMEM control condition was set at 1 and the other conditions were recalculated accordingly. (B) HCT8/E11 cells were treated with serum-free DMEM, CM^{CTRL} or CM^{DEX} for 24h, 48h, and 72h and subjected to an SRB assay. The serum-free DMEM control condition at 72h was set at 1 and the other conditions were recalculated accordingly. (A, B) Results are shown as the mean ± SD of three independent experiments and statistical analysis was performed using a one-way ANOVA and Tukey's multiple comparisons post-test. *: $p < 0.05$, ***: $p < 0.001$. (C) HCT8/E11 cells were treated with serum-free DMEM, CM^{CTRL} or CM^{DEX} and under those conditions, subjected to a cell morphology assay on collagen for 24h. Results are shown as scatter plots with means of at least three independent experiments and statistical analysis was performed using a Mann-Whitney test. **: $p < 0.01$, ***: $p < 0.001$. (D) HCT8/E11 cells were seeded in serum-free DMEM in Transwell inserts and the inserts were placed in wells containing DMEM, CM^{CTRL} or CM^{DEX}. After 24h migrated cells were stained with DAPI and the number of cells per microscopic field (10 x magnifications) was counted. Results are shown as box plots with the mean of three independent experiments, with whiskers indicating min and max values. Statistical analysis was performed using a Mann-Whitney test. **: $p < 0.01$. (E, F) HCT8/E11 cells were seeded on type I collagen-coated E-16 plates and treated with serum-free DMEM, CM^{CTRL} or CM^{DEX}. Cell adhesion was measured via cell impedance on an xCELLigence system for 60 minutes. The area under the curve (AUC) was calculated for each replicate of each condition. Results are shown as the mean ± SD of three independent experiments and statistical analysis was performed on AUC using a one-way ANOVA and Tukey's multiple comparisons post-test. *: $p < 0.05$, ***: $p < 0.001$.


A.
HCT8/E11-luc cell growth in presence of CM
Relative bioluminescent signal intensity
2.5
2.0
1.5
1.0
0.5
0.0
*
***
***
CM^CTRL - + -
CM^DEX - - +
x10^6 0.5 1.0 1.5
[photon/sec/cm²/steradian]
D.
HCT8/E11 cell migration
Number of migrateted cells
200
150
100
50
0
***
**
***
CM^CTRL - + -
CM^DEX - - +
B.
HCT8/E11 cell proliferation
Relative cell proliferation
3.5
3
2.5
2
1.5
1
0.5
0
DMEM
CM^CTRL
CM^DEX
***
***
***
24 48 72
Time [h]
E.
HCT8/E11 adhesion to collagen coating
Relative cell index
6
5
4
3
2
1
0
DMEM
CM^CTRL
CM^DEX
0 10 20 30 40 50 60
Time [min]
C.
HCT8/E11 cell morphology assay
Relative cell spread index
15
10
5
0
***
***
***
CM^CTRL - + -
CM^DEX - - +
F.
HCT8/E11 adhesion to collagen coating
AUC
500
400
300
200
100
0
ns
ns
ns
CM^CTRL - + -
CM^DEX - - +

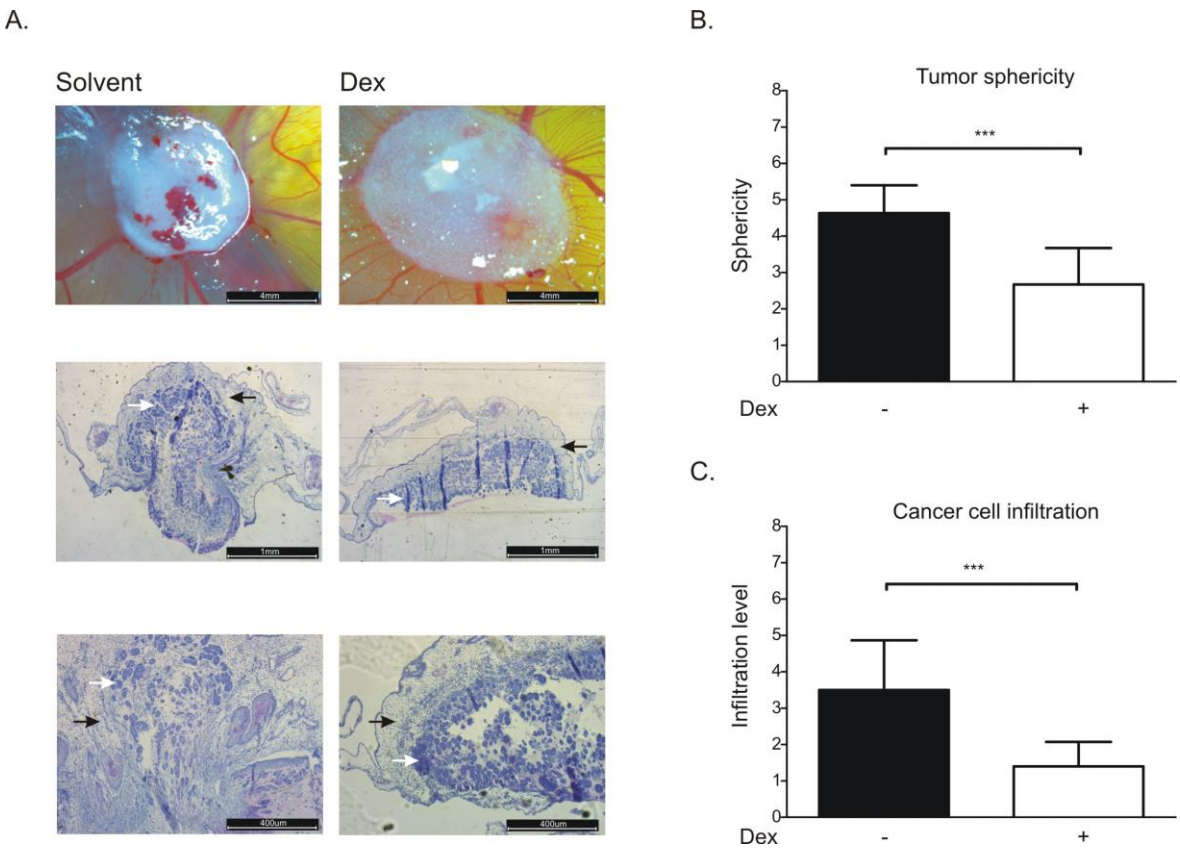


Figure 38. Dex treatment inhibits tumor formation *in vivo*. (A, B, C) HCT8/E11 cells and CT5.3hTERT CAFs were seeded in Matrigel drops on the CAM of 9 day-old chick embryos and treated with solvent or Dex (1µM) for 48h and then re-treated for another 72h. Five days post seeding, tumors were examined under the stereomicroscope, fixed, embedded in paraffin and subjected to hematoxylin-eosin staining. (A) Black arrows indicate CAM's mesenchyme; white arrows indicate clusters of cancer cells. Tumors were scored for (B) sphericity and (C) cancer cell infiltration in a scale from 1 to 5. Results (B, C) are shown as the mean ± SD of four independent experiments and statistical analysis was performed using an unpaired t-test. ***: $p < 0.001$.

3.4. Discussion

Recruited by cancer cells at the invasion front, stromal myofibroblasts, also known as cancer-associated fibroblasts (CAFs), are known to promote cancer progression [127]. Recent findings show that radiotherapy and chemotherapy affect cancer microenvironments, leading to the release of certain stromal-derived cancer-promoting factors and subsequent therapy resistance [142,159]. On that account, targeting the cancer environment forms an important strategy in cancer therapy [167]. In our study, we show that glucocorticoids (GCs), often given to patients during cancer treatment [278], could neutralize the cancer-promoting impact of CAFs on colon cancer cells.

Our model HCT8/E11 colon cancer cells do not express a functional glucocorticoid receptor (GR) (Figure 34). Without extra stimulation these cells display a round morphology on type I collagen matrix and limited migratory properties through a porous membrane (Addendum 3, Supplementary Figure 12, Figure 37C-D). We confirm here that colon cancer-derived CAFs can trigger aggressive behavior of cancer cells. CAFs' secreted factors stimulate growth and invasive morphology of HCT8/E11 cells (Figure 37A-C), corresponding with previous reports in various types of cancer cells [139,428-430]. Moreover, CAF conditioned medium (CM) facilitated HCT8/E11 motility by promoting cell migration through a porous membrane, which is also in line with previous findings [35,431,432].

In our previous study, we have shown that GCs have a significant effect on colon cancer-derived CAFs, modulating production of several molecules [389]. Following GC treatment, CAFs display an impaired expression and subsequent secretion of tenascin C (TNC) and hepatocyte growth factor/scatter factor (HGF/SF) [389]. Moreover, we have reported GC-mediated downregulation of transforming growth factor (TGF)β, urokinase-type plasminogen activator (uPA), angiopoietin-like protein-2 (ANGPTL2), diminished production of prostaglandins and also an increase in expression of angiogenin (ANG) [389,420]. These molecules are associated with cancer proliferation, invasion and/or angiogenesis [100,110,133,404,406,433]. In our current study, we extend these findings by showing that extracellular matrix (ECM) proteinase MMP-2's expression, and MMP-2's and MMP-9's potential activities are diminished in CAF CM after treatment with the GC dexamethasone (Dex) (Figure 35). Table 8 contains a list of GC-sensitive factors secreted by colon cancer-derived myofibroblasts and reported to date.

Our results show that GC-treated CAFs differently affect colon cancer cell proliferation, invasion, and motility, as compared to the control CAFs. Treatment with GCs resulted indirectly in impaired colon cancer cell proliferation (Figure 36A, 37A-B). Moreover, CM from Dex-treated CAFs (CM^{DEX}), had a significantly weaker potential to induce invasive morphotype of HCT8/E11 cells on collagen,

compared to CM from solvent-treated cells, CM^{CTRL} (Figure 37C, Addendum 3, Supplementary Figure 12). Furthermore, in the *in vivo* chick chorioallantoic membrane (CAM) model, HCT8/E11 colon cancer cells applied together with CT5.3hTERT CAFs formed spherical, invasive tumors. In line with the *in vitro* results, treatment with Dex led to a decreased cancer cell infiltration into the CAM's mesenchymal layer, as well as to lack of tumor sphericity (Figure 38A-C). Lack of a functional GR in HCT8/E11 cells suggests that Dex-induced growth inhibition and impaired invasion originate from the added CAFs, which upon Dex treatment secrete a modified cocktail of factors, resulting in inadequate growth- and invasion-stimulatory signals compared to the solvent-treated CAFs.

Table 8. List of GC-sensitive factors detected in colon cancer-derived CAFs' secretome in current and previous studies (c.s. – current study).

Factor	GC-mediated effects
Angiogenin	Increased mRNA and protein levels [420]
ANGPTL-2	Decreased mRNA and protein levels [420]
HGF/SF	Decreased mRNA and protein levels [389]
MMP-2	Decreased protein levels and potential activity [c.s.]
MMP-9	Decreased potential activity [c.s.]
Prostaglandins ($PGF_{2\alpha}$, PGI_2, PGE_2)	Decreased concentration [420]
tenascin C	Decreased mRNA and protein levels [389]
TGFβ	Decreased mRNA and protein levels [389]
uPa	Decreased mRNA and protein levels [420]

Interestingly, CM^{DEX} seemed to favor HCT8/E11 cell migration through a porous membrane to a greater extent as compared to CM^{CTRL} (Figure 37D). Cell migration mechanisms depend greatly on the cell type and surrounding tissue environment [111]. Cells with a round morphology, unlike spindle-shaped, elongated cells, migrate by adapting their shape, which enables them to squeeze through gaps or narrow spaces [111]. The fact that CM^{DEX} has an impaired ability to promote a spread morphotype of HCT8/E11 cells on collagen as compared to CM^{CTRL} (Figure 37C, Addendum 3, Supplementary Figure 12), might be a cause of different migration efficiencies between CM^{DEX} and CM^{CTRL} treatments. Moreover, the induction of invasive morphotype might be affected by decreased MMP-2 protein levels and potential protein activity in the CM^{DEX}, as compared to CM^{CTRL} (Figure 35A-D). MMP-2 is known to affect cell motility via cleavage of adhesion molecules [434] and via proteolytic degradation of matrix proteins, limiting cell-surface interactions [435]. In line, MMP-2, but not MMP-9, was previously reported to cleave type I collagen [436]. Nevertheless, neither in co-culture system nor in experiments with use of CAF-derived CM we detected indirect Dex-mediated effects on colon-cancer cell adhesion (Figure 36B, 37E-F).

Results displaying decreased pro-invasive potential of Dex-treated CAFs correspond with decreased amounts of secreted TNC and HGF in the CM^{DEX} [389], factors previously reported as important stimulators of HCT8/E11 colon cancer cell invasion via RhoA and Rac pathways [77]. Moreover, treatment of CAFs with other GCs, resulted in a similar TNC and HGF decrease [389], and use of CM from such treated CAFs caused also a diminished invasive morphotype in HCT8/E11 cells (Addendum 3, Supplementary Figure 13A), suggesting that the observed effect is universal for other GCs. Interestingly, a selective GR modulator (SEGRM), compound A (CpdA), only slightly affected TNC and HGF protein levels [389], and in line, the impact of CM^{CPDA} on HCT8/E11 morphology was not different from CM^{CTRL} (Addendum 3, Supplementary Figure 13B). Since CpdA triggers solely GR-transrepression, these results point to a conclusion that TNC's and HGF's downregulation is caused by GR-transactivation mechanisms [268]. Although our data and literature support give a strong argument that GC-mediated decrease of TNC and HGF in CAF-derived CM could be responsible for diminished pro-invasive effects of the CM, as well as the impaired cancer cell invasion in the CAM model, the addition of recombinant TNC and HGF proteins to CM^{DEX} did not restore the invasive morphotype up to the CM^{CTRL} levels (Addendum 3, Supplementary Figure 14A). These data suggest that HGF and TNC are not sole players in the observed phenomena and point to the co-involvement of other factor(s) sensitive to GC treatment.

Cancer therapy, as most treatments, has associated side effects. It is documented that radiotherapy and chemotherapy lead to activation of stroma, subsequently resulting in environment-mediated drug resistance (EMDR) and protection of cancer cells against treatment [142,159]. Lotti, *et al.* described that chemoresistance of colorectal cancer-initiating cells was increased by IL-17A produced by chemotherapy-induced CAFs [162]. Moreover, factors secreted by CAFs stimulate nuclear translocation of AKT, survivin, and MAPK p38 in colorectal cancer cells, leading to protection against chemotherapeutics oxaliplatin and 5-fluorouracil [164]. Therefore, a potential solution to overcome EMDR is required. In advanced pancreatic cancer neutralizing effects of nab-paclitaxel treatment on activated stroma were observed, which was manifested by decreased tumor stiffness and as such, disrupted collagen architecture and a decrease in CAF density [437]. Furthermore, in our recent study we have reported that GCs reduced the pro-angiogenic abilities of colon cancer-derived CAFs, which was reflected by a lack of pro-migratory stimuli on endothelial cells, as compared to the control [420]. Lastly, in this current study, we show that Dex-treated CAFs have additionally an impaired ability to promote cancer cell growth and invasion, as compared to the non-treated CAFs. These results suggest that GCs could be helpful in neutralizing the negative effects of activated stroma and possibly also EMDR.

However, as reported before, around 50% of colon cancer tumors express GR [312]. In this current study, we used a model of GR-irresponsive HCT8/E11 cancer cells, which allowed us to limit GC-mediated effects to CAFs (*in vitro*) and other stromal cells (*in vivo*). However, the effects of GCs on GR-responsive cancers must certainly also be taken into account. Recent studies in various cancer cell lines, surgical resections and xenografts revealed GC-mediated protection of cancer cells against the cytotoxic therapies. The mechanism behind this therapy resistance was linked to GC-driven, most probably GR-mediated protection from apoptosis [299]. Nevertheless, the beneficial aspects of GC-treatment in cancer cells were also reported. In the glioblastoma cells, Dex decreased MMP-2 secretion and invasiveness of these cells via an MKP-1-mediated mechanism [291]. Similar anti-invasive properties of GC treatment were observed in bladder cancer cells and were accompanied by reduced expression of MMP-2, MMP-9, IL-6 and VEGF. Although the anti-apoptotic properties of GCs were also noted in case of these cells, in the *in vivo* model GC-treated tumors were in general less aggressive [297]. Moreover, in two recent studies GCs were shown to counteract TGFβ- and hypoxia-induced EMT in colon cancer cells [309,310]. Therefore, it seems that depending on the target cells, GCs can have different effects ranging from detrimental to positive, which points to the importance of an individual approach in planning cancer treatment.

In conclusion, our findings show that GCs, besides their present role during cancer therapy, might have an additional beneficial effect in colon cancer treatment via their impact on the activated stroma. GCs could neutralize the negative, pro-aggressive effects of CAFs on cancer cells, by modulating factors secreted by these cells. These combined factors contribute, directly or indirectly but collectively, to observed effects on cancer cell growth and invasiveness. Therefore, further studies on the endogenous and treatment-affected CAF secretomes are needed to decipher this complex mechanism.

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CHAPTER 4: ARTICLE 4

Impact of a plant-derived selective glucocorticoid receptor modulator, compound A, on endothelial cells and angiogenesis.

Zuzanna Drebert[1,2], Mark MacAskill[3], Karolien De Bosscher[2,4], Marc Bracke[1,2], Patrick W.F. Hadoke[3*], Ilse M. Beck[1,2, 5*]

[1] Laboratory of Experimental Cancer Research, Department of Radiation Oncology & Experimental Cancer Research, Ghent University, Ghent, Belgium

[2] Cancer Research Institute Ghent (CRIG), Ghent, Belgium

[3] University/BHF Centre for Cardiovascular Science, The Queen's Medical Research Institute, University of Edinburgh, Edinburgh, United Kingdom

[4] Receptor Research Laboratories, Nuclear Receptor Lab, Medical Biotechnology Center, VIB Department of Biochemistry, Ghent University, Ghent, Belgium

[5] Department Health Sciences, Odisee University College, Ghent, Belgium

[*] Shared last authorship

Manuscript in preparation

Abstract

Angiogenesis, a process of vessel formation, is crucial for embryo development and tissue recovery, but also plays a role in pathological conditions, such as cancer, retinopathy, and tissue ischemia. Glucocorticoids (GCs), steroidal ligands of the glucocorticoid receptor (GR), are well-documented agents with anti-inflammatory and angiostatic properties, and are therefore, widely used in the clinic. However, long-term GC treatment is associated with detrimental side effects, such as diabetes and osteoporosis, propagating the search for alternative and more selective GR modulators (SEGRM). A plant-derived SEGRM, compound A (CpdA), has a non-steroidal structure and was reported to trigger GR-mediated transrepression but not transactivation. This latter mechanism is partially associated with adverse clinical effects of GCs.

In the current study, we compared the angiostatic and anti-inflammatory properties of a synthetic GC dexamethasone (Dex) with the SEGRM CpdA in endothelial cells (ECs). Unlike Dex, CpdA caused an impaired translocation of GR in human umbilical vein ECs (HUVECs), but interestingly, its anti-inflammatory efficiency was more pronounced in comparison to the GC. *In vitro* studies showed that CpdA, unlike Dex, had mild growth-inhibitory effects on HUVECs, however, neither compound had a strong impact on endothelial characteristics of HUVECs or on the mRNA expression of several angiogenesis-related genes. Nevertheless, in an *ex vivo* aortic ring assay, Dex strongly inhibited neovessel outgrowth, while CpdA's impact was not different from the control stimulation. Our study confirms Dex's angiostatic properties and suggests that these effects may originate from GR transactivation events.

4.1. Introduction

The glucocorticoid receptor (GR) belongs to the nuclear receptor family and is expressed in almost every human tissue [174]. GR regulates many important biological processes involved in development, metabolism, and immune responses [172]. Glucocorticoids (GCs), act as steroidal ligands of GR that bind to the receptor in the cytoplasm, which leads to the protein's conformational change, a release from its chaperone complex and a subsequent GR nuclear translocation [220]. In the nucleus, GR acts as a transcription factor, where as a homodimer it activates transcription of multiple genes (GR transactivation), or as a monomer it can suppress transcription of genes via tethering to another transcription factor (GR transrepression). Other nuclear mechanisms along with non-genomic actions of GR have also been described [188]. Although GR's ability to suppress the transcriptional activity of nuclear factor (NF)κB and activator protein (AP)-1 reflects a main anti-inflammatory strategy, GR transactivation and non-genomic activity were also proven to play a role in the inhibition of inflammation [231]. Because of their anti-inflammatory properties, GCs are widely used in the clinic against autoimmune and inflammatory afflictions [204]. Due to their pro-apoptotic abilities towards blood cells, GCs are also used in therapy of hematological malignancies, such as leukemia and multiple myeloma [279]. Moreover, GCs' angiostatic properties are also well-documented [333,334,397] and used in the therapy of infantile hemangiomas [276]. Furthermore, GCs are used in the clinic for the treatment of solid tumors, as part of chemo- or hormonal therapy in breast and prostate cancer or applied as an adjuvans to relieve side effects of chemotherapy in various cancer treatments [278]. However, long-term administration or high-dose treatment with GCs may result in several adverse effects, such as diabetes, osteoporosis, hypertension, and immunosuppression [209], but also induction of chemotherapy resistance [299]. Moreover, the occurrence of GR resistance (hyposensitivity to GCs) has been documented to negatively influence the outcome of GC therapy [174,204]. These drawbacks have triggered a search for alternative solutions including selective GR agonists and modulators (SEGRAMs) [262,438].

The idea behind SEGRAMs stems from the simplified hypothesis that most of GR-mediated therapeutic properties derive from GR transrepressive actions and undesirable side effects are mostly a result of GR transactivation [256]. Several dissociated GCs or selective GR agonists (SEGRAs), such as RU24858, were originally shown to favor GR transrepression over transactivation *in vitro*, however, these results did not translate to the *in vivo* situation [257,439]. Selective GR modulators (SEGRMs), compounds lacking steroidal structure, yet capable of binding GR and triggering solely GR transrepression, are of an even bigger interest [255]. Different SEGRMs have different origins, therefore their characteristics cannot be generalized. A SEGRM

with a very simple structure, compound A (CpdA), is an aziridine derivative isolated from a Namibian shrub *Salsola tuberculatiformis* [267]. CpdA was proven to bind GR, however in a yet unidentified manner, and to trigger a GR conformational change that differs from GC-GR binding. Such CpdA-activated GR is unable to form homodimers, and therefore, acts preferentially as a monomer to initiate GR transrepression, which leads to suppression of multiple pro-inflammatory genes expression [268,269,440]. In line, various mice models for inflammation revealed a restricted side effect profile due to CpdA treatment, as compared to synthetic GC dexamethasone (Dex) [268,269,275,441,442].

SEGRAMs are investigated not only in terms of inflammatory afflictions, but also to improve cancer treatment, and as such, RU24858, CpdA, avicin D and 21OH-6,19OP are SEGRAMs with a cancer-modulating potential [438]. RU24858's anti-tumor properties were shown in the SENCAR skin tumor model, as RU24858 similar to GCs reduced epidermal hyperplasia after induction with a tumor promoter TPA [443]. The SEGRA 21OH-6,19OP holds similar anti-inflammatory properties as classic GCs, however, it does not trigger chemotherapy resistance in a breast cancer model [444]. Avicin D, a plant-derived saponin, induced GR-mediated suppression of inflammation, but was also identified to induce apoptosis in various cancer cell lines. However, this pro-apoptotic mechanism is most likely not GR-dependent [445]. CpdA, besides its anti-inflammatory characteristics, was also shown to induce apoptosis in various cancer cell lines [351,353,446]. Moreover, in prostate cancer cells CpdA was able to interact with the androgen receptor (AR) and to inhibit its activity [353,447].

Angiogenesis is one of the hallmarks of cancer, and as such it is also a target in cancer therapy [8]. Angiogenesis results in the formation of new blood vessels from the pre-existing vasculature and is crucial during tissue development and repair [85]. Impaired angiogenesis may cause ischemic chronic wounds, which leads to tissue damage or dysfunction due to shortage of oxygen and nutrients [384]. Excessive angiogenesis, on the other hand, plays an important role in pathological states, such as malignant or inflammatory afflictions [44]. Although SEGRAMs' direct impact on cancer cells has gained interest and attention, SEGRAMs' effects on angiogenesis were not yet researched in detail, while GCs' angiostatic properties are well-established [448]. In this study, we attempted to evaluate the direct impact of a SEGRM CpdA on angiogenesis in comparison to a classical GC Dex, using models of human umbilical vein endothelial cells (HUVECs) and murine aortic rings.

4.2. Materials & Methods

4.2.1. Cells and reagents

Primary human umbilical vein endothelial cells (HUVEC; Promocell, Heidelberg, Germany) were cultured in Endothelial Cell Growth Medium-2 (EGM2; Lonza, Wokingham, UK), supplemented with 2% FCS, 0.1% VEGF, 0.4% hFGF-2, 0.1% R3-IGF-1, 0.1% hEGF, 0.1% ascorbic acid, 0.1% heparin, 0.1% GA-100, but omitting the addition of the supplied hydrocortisone. HUVECs were cultured at 37°C, 5% CO_2, on 0.1% gelatin-coated flasks. The cells were used between passages 2 and 7.

Dexamethasone (Dex) was purchased from Sigma-Aldrich (Dorset, UK, cat no: D1756), dissolved in ethanol and used at a final concentration of 1µM. A selective GR modulator (SEGRM), compound A (CpdA) was prepared according to [268] and used at a final concentration of 5µM. The total ethanol concentration (maximally 0.05%) was kept constant in all conditions in all experiments.

Recombinant murine tumor necrosis factor (TNF)α was produced and purified as described [355]. TNFα was dissolved in medium and used at final concentration of 2000IU/ml.

4.2.2. Cell viability and proliferation assays

In order to test HUVEC viability and metabolic activity, cells were seeded in 96-well plates (1.5 $x10^4$ cells/well) and left to adhere. Next, the confluent cell cultures were treated with solvent, Dex (1µM) or CpdA (5µM) for 24h and the cell viability was assessed with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [393] using reagents purchased from Sigma-Aldrich.

To assess HUVEC proliferation, HUVEC (4 $x10^3$/well) were seeded in 96-well plates, and treated the next day with solvent, Dex (1µM) or CpdA (5µM) for 24h, 48h, and 72h. After selected time points cells were fixed and subjected to a sulforhodamine-B (SRB) assay, according to [394].

Results were obtained with a Molecular Devices OPTImax Microplate Reader and the SoftMax® Pro 3.0 software. Data were expressed on a scale where maximal viability (MTT assay) or proliferation (SRB assay, at 72h post treatment) in solvent controls was set at 100%.

4.2.3. Indirect immunofluorescence

Indirect immunofluorescence microscopy was performed according to previous protocols [268]. Briefly, HUVECs were seeded on 0.1% gelatin-coated glass coverslips and treated the next day with solvent, Dex (1µM) or CpdA (5µM) for 2h. Subsequently, cells were washed with PBS, fixed with 2% paraformaldehyde, permeabilized with ice-cold acetone, blocked with 1% BSA, washed again with PBS and probed overnight at 4°C with a primary anti-GR antibody (1/200, anti-GR H-

300 antibody, Santa Cruz Biotechnology, cat no: sc-8992). Subsequently, cells were exposed to Alexa Fluor 488 goat anti-rabbit secondary antibody (1/800, Invitrogen Molecular Probes, cat no: A11008) for 2h at room temperature, and lastly, stained with DAPI (0.4 µg/ml) in order to visualize the nuclei. Observations and image processing were performed using a fluorescence microscope (40 x magnification, Axioscope, Zeiss, Oberkochen, Germany), CoolSNAP camera (Photometrics, AZ, USA) and MCID Basic 7.0 software.

4.2.4. Tube-like structure (TLS) formation assay

The evaluation of TLS formation was performed by seeding HUVECs (1.5 x10^4 cells/well) onto Matrigel™ (Corning, Flintshire, UK)-coated 96-well plates, as previously described [395]. Cells were treated with solvent, Dex (1µM) or CpdA (5µM) for 6h prior to microscopic evaluation (5 x magnification, phase contrast microscope, CoolSNAP camera, MCID Basic 7.0 software). The further analysis of TLS formation (total tube length) was performed with the Angiogenesis Analyzer plug-in [396] developed for ImageJ software [362].

4.2.5. RNA isolation and RT-qPCR

HUVECs were treated with solvent, Dex (1µM) or CpdA (5µM) for 24h. After washing with PBS, cells were subjected to total mRNA isolation using an RNeasy Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Reverse transcription (RT) of HUVEC RNA was performed using QuantiTect Reverse Transcription Kit (Qiagen) and the obtained cDNA was subjected to quantitative PCR (qPCR) using LightCycler 480 SYBR Green I Master reagents (Roche Diagnostics, Rotkreuz, Switzerland), according to the manufacturer's instructions. qPCR reactions, performed in triplicates, were run using the LightCycler 480 system (Roche Diagnostics) applying the following conditions: (A) initial denaturation 95 °C, 5min; (B) 45 cycles of denaturation 95°C, 15s, annealing and elongation 60 °C, 45s. Specific signal obtained for the gene of interest was normalized to the respective geometric mean signal for three reference genes, namely GAPDH, PPIB, and 36B4. Primer sequences are listed in Addendum 5 (Supplementary Table 4).

4.2.6. Acetylated low density lipoprotein (Ac-LDL) uptake assay

HUVECs were seeded onto 0.1% gelatin-coated glass coverslips and incubated overnight. Next, cells were treated with solvent, Dex (1µM) or CpdA (5µM) for 24h. Ac-LDL assay uptake was performed as described previously [449]. In short, HUVECs were incubated with 5µg/ml Ac-LDL conjugated with alexa-488 (Invitrogen, Glasgow, UK, cat no: L23380) for 5h. Subsequently, cells were washed with PBS prior to fixation with 2% paraformaldehyde and washed again with PBS. To visualize the nuclei, cells were stained with DAPI (0.4 µg/ml). Images, taken using a fluorescence microscope (Axioscope, Zeiss), CoolSNAP camera (Photometrics), and MCID Basic 7.0 software,

were further analyzed for fluorescence signal intensity for a number of cells per image with ImageJ software [362].

4.2.7. Aortic ring assay

For the *ex vivo* aortic ring assay [397] we used 8-12 weeks-old C57BL/6 male mice (Charles River Laboratories). After the animals were sacrificed (via CO_2 asphyxiation) the thoracic aortas were isolated, the adjacent tissue cleaned off and washed with serum-free DMEM. Next, aortas were divided into 1-2mm rings and embedded in type I collagen (1mg/ml, Sigma-Aldrich). After 1h explants were treated with solvent in 2% FCS DMEM (positive control), Dex (1µM) or CpdA (5µM) in 2% FCS DMEM, or with solvent in serum-free DMEM (negative control), at 37°C, 5% CO_2. Media and treatments were replaced after 3 and 7 days in culture. Neovessel outgrowths were counted on days 5, 7, and 10 using phase-contrast microscopy.

4.2.8. Statistical analyses

Data are presented as mean ± standard deviation or as a scatter plot with mean. We performed the statistical analyses using GraphPad Prism 5.03 with a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test, or with a Mann-Whitney U test. The applied test is included in the figure legends. A $p<0.05$ was considered statistically significant. Results were expressed as a relative number, where the control condition was set at 1, 100 or 100% and other conditions were recalculated respectively.

4.3. Results

4.3.1. Effects of CpdA and Dex on GR translocation, transactivation and transrepression

The GR upon binding to its steroidal ligand, is expected to be driven from the cytoplasm to the nucleus [220]. Indirect immunofluorescence imaging performed after 2h of treatment has shown that in HUVECs CpdA (5µM) had an impaired ability to translocate GR to the nucleus, as compared to the GC Dex (1µM) (Figure 39A). Furthermore, Dex and CpdA displayed little to no impact on GR's mRNA expression (Figure 39B). As expected, CpdA, unlike Dex, did not upregulate GILZ. This result confirms CpdA's lack of GR-transactivating properties, which are characteristic for the steroidal ligands of the receptor (Figure 39C). A pro-inflammatory molecule TNFα stimulated mRNA expression of several inflammatory markers in HUVECs, namely the interleukin (IL)-1β, the chemokine RANTES (Regulated on Activation, Normal T cell Expressed and Secreted, also known as CCL5) and TNFα itself. Further treatment with Dex and CpdA strongly suppressed IL-1β mRNA expression (Figure 39D), however, mRNA levels of RANTES and TNFα were only suppressed in HUVEC following CpdA treatment (Figure 39E, F). Overall, Dex and CpdA behaved as expected and both ligands were shown to be functional in HUVECs.

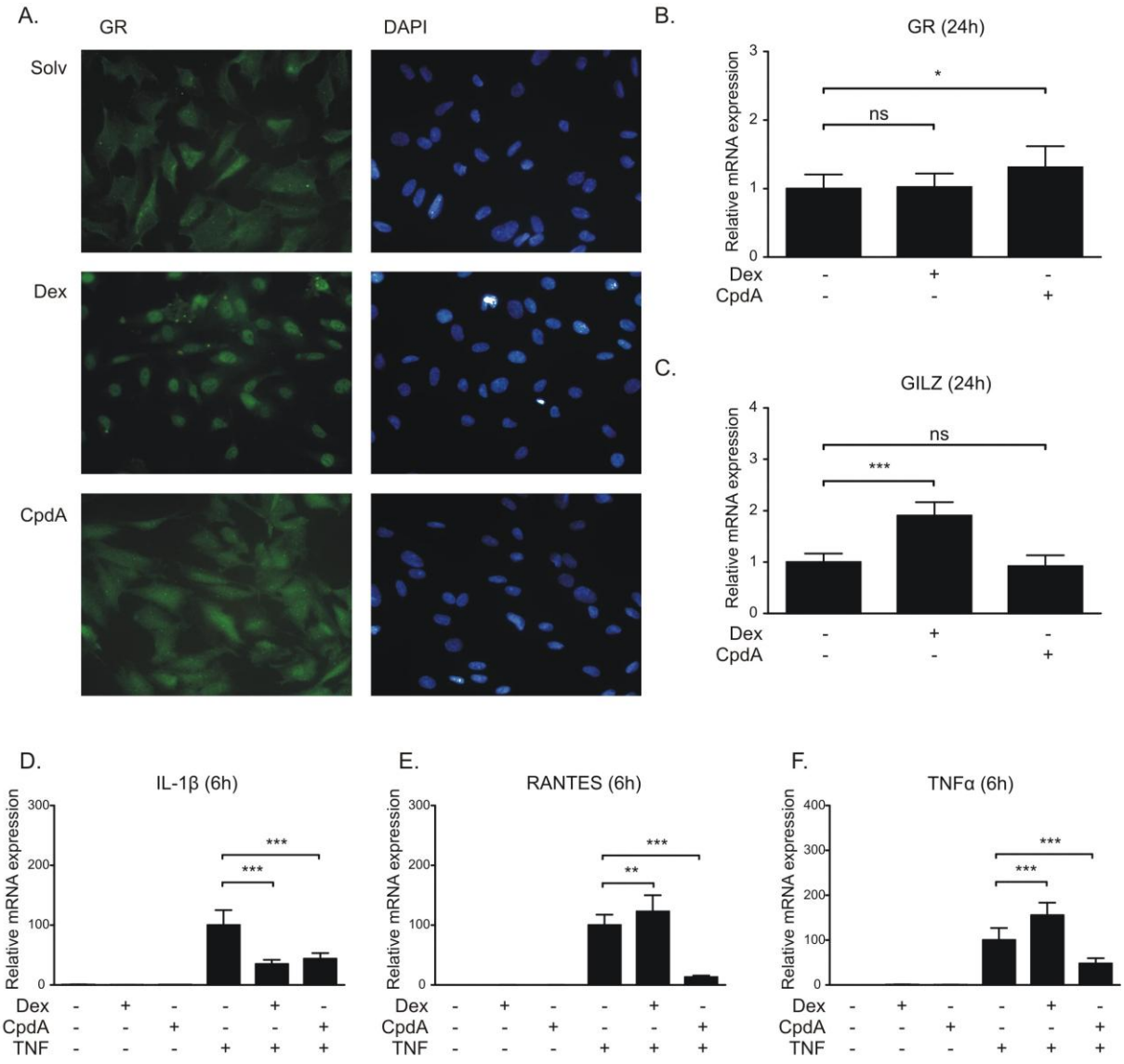

Figure 39. CpdA displays an impaired GR-translocation ability, but strong inflammation-suppressive actions in HUVEC. (A) HUVECs were seeded on 0.1% gelatin-coated coverslips, left overnight to adhere and subsequently treated with solvent, Dex (1µM) or CpdA (5µM) for 2h. After washing with PBS, fixing with 2% paraformaldehyde and permeabilizing the cells, indirect immunofluorescence was performed to detect GR. DAPI staining indicates the nuclei. Magnification: 40 x (B, C) HUVECs were treated with solvent, Dex (1µM) or CpdA (5µM) for 24h or (D, E, F) for 1h and either or not co-treated with TNFα (2000IU/ml) for another 5h. Total mRNA was subjected to RT-qPCR assaying (B) GR, (C) GILZ, (D) IL-1β, (E) RANTES, and (F) TNFα mRNA levels, and results were normalized to the respective geometric mean of GAPDH, PPIB, and 36B4 household genes' mRNA levels. The condition induced with solvent (B, C) was set as 1, alternatively the condition induced with TNFα (D, E, F) was set at 100 and other results were recalculated accordingly. Results (B, C, D, E, F) shown are the means ± SD of three independent experiments and statistical analysis was performed using a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test. ns: not significant, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

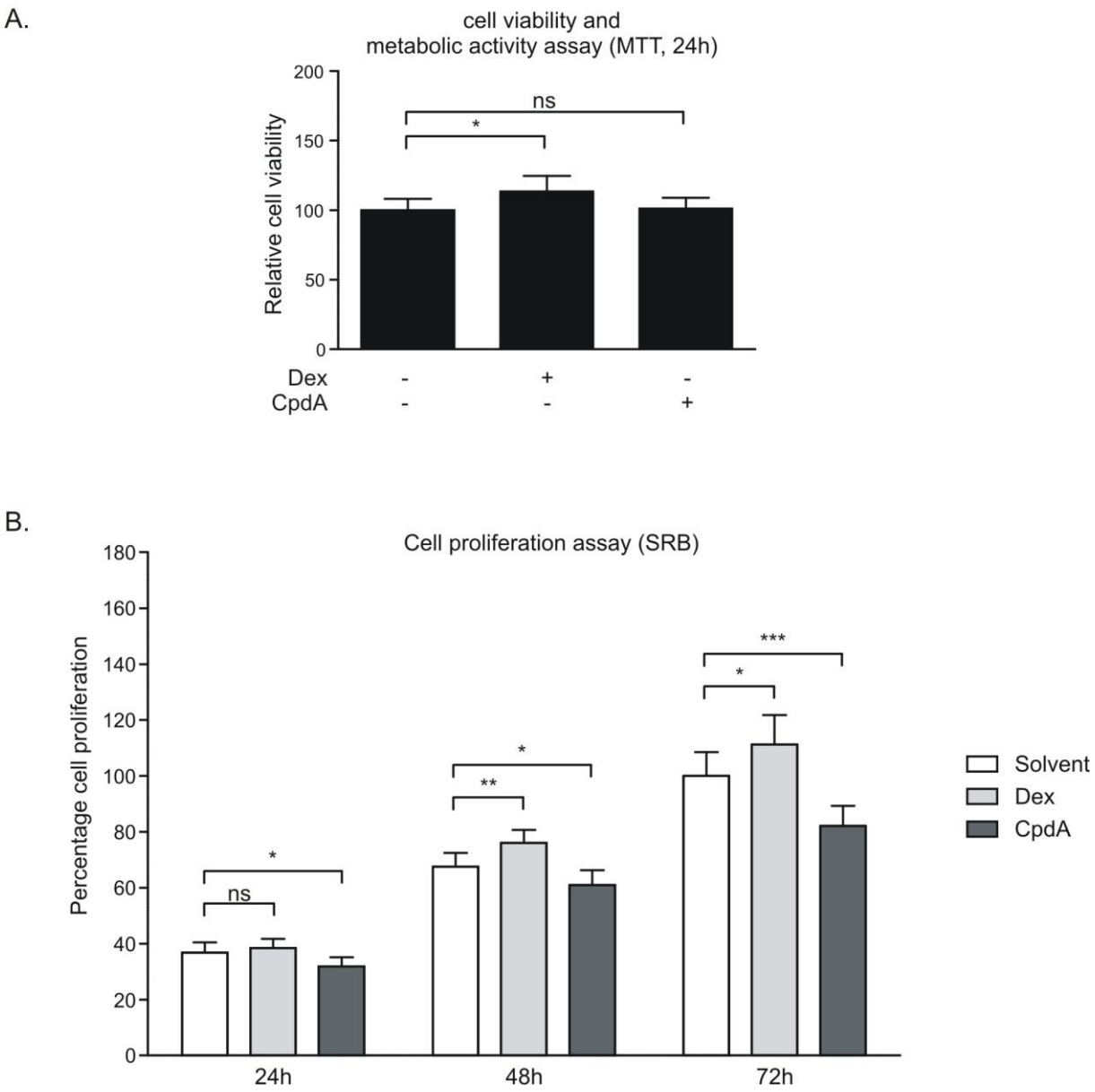

Figure 40. CpdA slightly inhibits HUVEC growth, but does not affect cell viability. (A) Confluent cultures of HUVECs were treated with solvent, Dex (1µM) or CpdA (5µM) for 24h and subsequently subjected to an MTT assay to assess percentage cell viability. Obtained values were normalized to the control treatment (solvent). (B) HUVECs were treated with solvent, Dex (1µM) or CpdA (5µM) for 24h, 48h or 72h and subjected to an SRB assay. Percentage cell proliferation was calculated and obtained values were normalized to a control treatment (solvent) at 72h, which indicates a maximal cell proliferation. Results (A, B) are displayed as mean ±SD of three independent experiments and statistical analysis was performed using a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test. ns: not significant, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

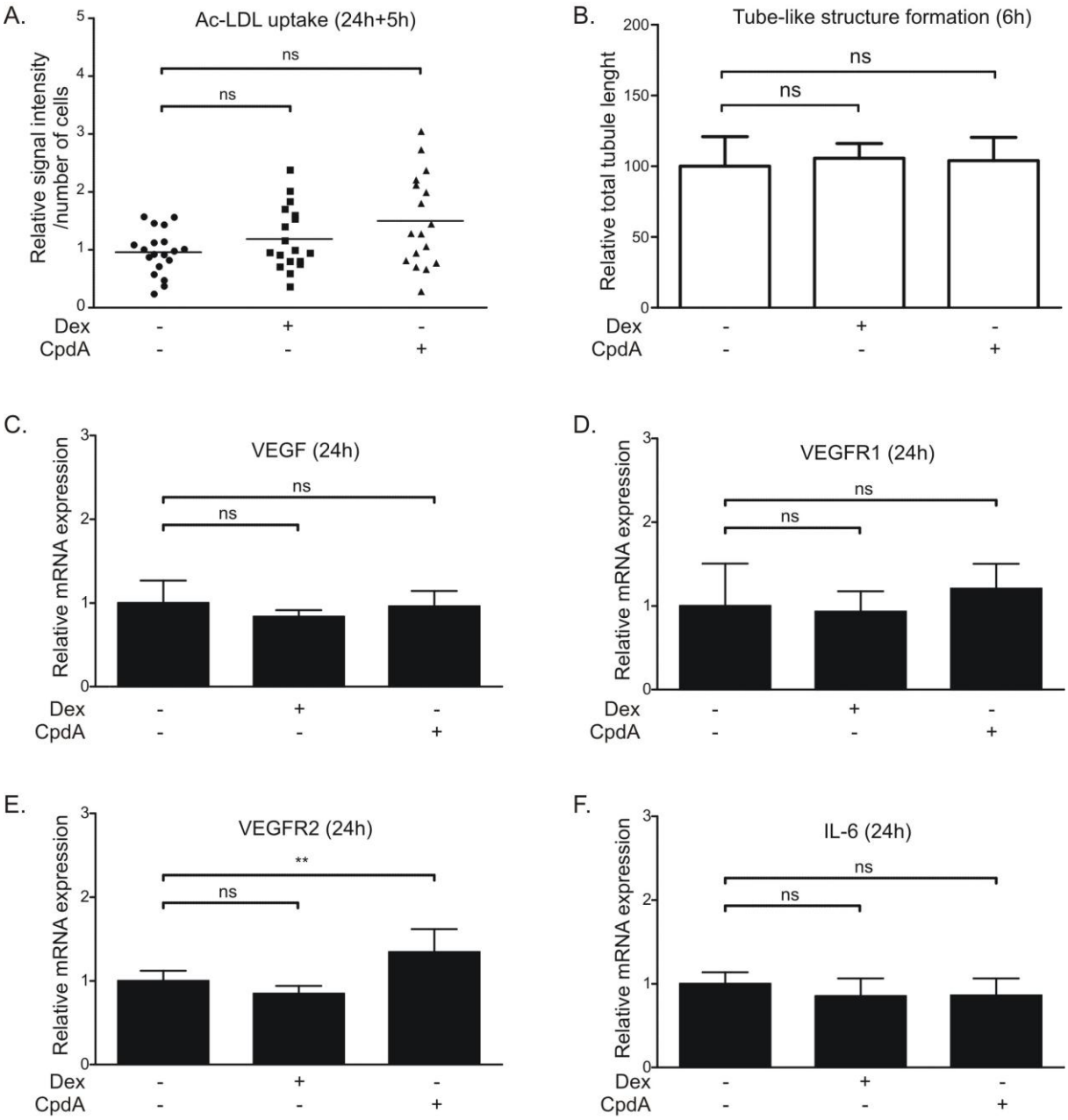

Figure 41. CpdA does not affect endothelial characteristics of HUVECs. (A) HUVECs, seeded onto coverslips were treated for 24h with solvent, Dex (1µM) or CpdA (5µM). Next, Ac-LDL conjugated with Alexa-488 (5µg/ml) was added to the cells. After 5h cells were washed, fixed and counterstained with DAPI prior to obtaining the microscopic images. Fluorescence signal intensity was evaluated using ImageJ software and divided over the number of cells assessed. Scatter plots represent data of three independent experiments. Results were analyzed using a Mann-Whitney U test. ns: not significant. (B) HUVECs were seeded on Matrigel-coated wells and treated with solvent, Dex (1µM) or CpdA (5µM). After 6h phase-contrast images were taken and total tubule length was assessed using the Angiogenesis Analyzer tool for ImageJ software. (C, D, E, F) HUVECs were treated with solvent, Dex (1µM) or CpdA (5µM) for 24h. Total mRNA was subjected to RT-qPCR assaying (C) VEGF, (D) VEGFR1, (E) VEGFR2, and (F) IL-6 mRNA levels. Results were normalized to the respective geometric mean of GAPDH, PPIB, and 36B4 household genes' mRNA levels. The condition induced with solvent was set at 1 and other results were recalculated accordingly. Results (B, C, D, E, F) shown are the means ± SD of three independent experiments and statistical analysis was performed using a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test, ns: not significant, **: $p<0.01$.

4.3.2. Impact of Dex and CpdA on HUVEC viability and proliferation

Analysis of the cell viability and metabolic activity assays (MTT) revealed that neither Dex (1μM) nor CpdA (5μM) had a cytotoxic effect on a confluent monolayer of HUVECs after 24h treatment (Figure 40A). In contrast, Dex caused a slightly higher production of the formazan salt compared to the solvent control. In the cell proliferation assay (SRB), we observed that CpdA impaired HUVEC growth compared to the solvent treatment (Figure 40B) which was manifested from a 24h treatment onwards. Treatment with Dex, on the other hand, had a positive impact on HUVEC growth, as detected upon 48 and 72h.

4.3.3. Effects of Dex and CpdA on the endothelial characteristics of HUVECs

A series of experiments were performed in order to establish a potential impact of the SEGRM CpdA on the biology and endothelial character of HUVECs. Neither Dex (1μM) nor CpdA (5μM) influenced HUVECs' ability to internalize acetylated low density lipoprotein (Ac-LDL) (Figure 41A), a feature characteristic for endothelial cells (ECs) [449]. Another typical EC ability, formation of tube-like structures (TLS) on a gel matrix (Matrigel), was not affected following treatment with either Dex or CpdA, which was reflected in a lack of differences in lengths of TLSs, as compared to solvent control (Figure 41B). Representative images of the Ac-LDL uptake assay and TLS assay are shown in Addendum 4 (Supplementary Figures 15 and 16, respectively). mRNA expression of factors related to angiogenesis, namely vascular endothelial growth factor (VEGF, Figure 41C), VEGF receptor 1 (VEGFR1, Figure 41D) or IL-6 (Figure 41F) did not reach a significant difference after 24h treatment with either Dex or CpdA. RT-qPCR analysis of VEGFR2 has revealed that CpdA treatment caused a slight (1.34 fold change) upregulation of the receptor's mRNA (Figure 41E).

4.3.4. Effects of CpdA and Dex on angiogenesis in *ex vivo* aortic ring assay

Murine aortic rings treated with medium supplemented with 2% FCS and solvent displayed outgrowths of several neovessels, compared to the negative control (serum-free conditions), where few or no outgrowths were identified. Exposure to Dex (1μM) caused a strong inhibition of neovessel formation observed after 5, 7, and 10 days of treatment, while stimulation with CpdA (5μM) had no inhibitory effects (Figure 42A, B, C, D), as compared to the positive control (medium supplemented with serum).

4.4. Discussion

Because of their anti-inflammatory properties glucocorticoids (GCs) are used in the clinic since 1950s [204]. GCs are potent drugs with a wide spectrum of action and they are also known for their pro-apoptotic abilities in hematological cells, as well as for angiostatic properties [279,333]. Nevertheless, the use of GCs is associated with adverse effects, the occurrence of GC resistance

and, in case of certain solid tumors, an induction of chemotherapy resistance [204,209,299]. Selective glucocorticoid receptor modulators (SEGRM) are compounds that trigger only a portion of the actions normally associated with GCs, therefore, in the future SEGRM might serve as alternatives for steroidal compounds in particular treatments [256]. Several SEGRMs are under intensive investigation, however, their role in the process of neovessel formation (angiogenesis), is not yet described.

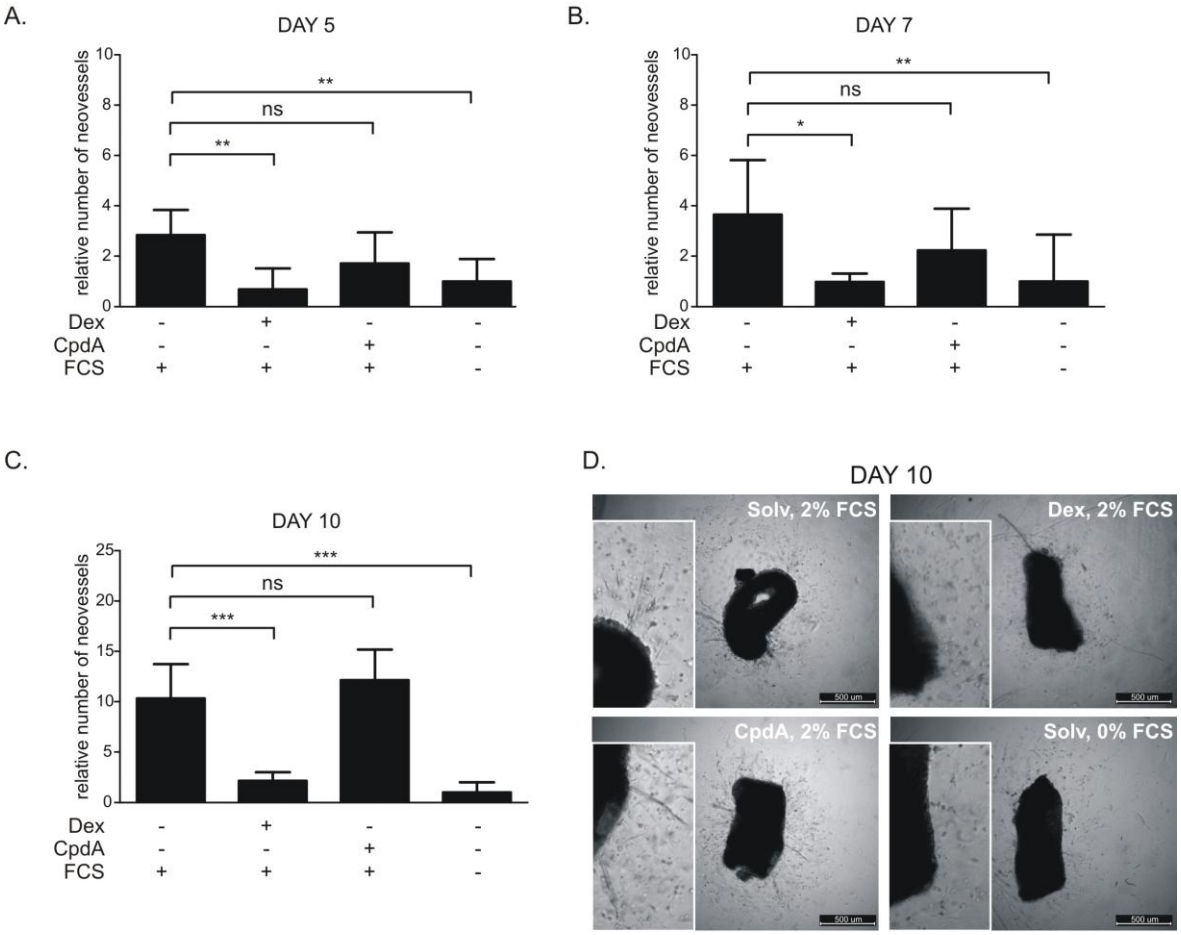

Figure 42. CpdA, in contrast to Dex, does not inhibit neovessel growth in the aortic ring assay. (A, B, C, D) Aortic rings were prepared from aortas isolated from adult male C57BL/6 mice. Explants were embedded in collagen and treated with solvent, Dex (1µM) or CpdA (5µM) in DMEM supplemented with 2% FCS, or treated with solvent in serum-free DMEM. Neovessel sprouts were quantified after 5 days (A), 7 days (B), and 10 days (C, D) in culture. (A, B, C) Histograms represent the mean ± SD of three independent experiments. Results were analyzed using a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test. ns: not significant, *: p<0.05, **: p<0.01, ***: p<0.001.

GCs have a well-documented history of angiogenesis inhibition, and are used as angiostatics in infantile hemangiomas and in ocular afflictions [276,334], however, the mechanism behind these actions is not well-defined. Application of GCs resulted in diminished angiogenesis in several cancer-based studies. Indirect effects on angiogenesis were described via GC-mediated downregulation of VEGF in cancer cells [287,336], but also via neutralization of the pro-angiogenic impact of cancer-associated fibroblasts (CAFs) in a colon cancer model [420]. Moreover, a direct

GC-mediated growth inhibitory effect was reported in vascular smooth muscle cells [386] and other reports describe direct anti-angiogenic effects of GCs on endothelial cells (ECs) [344] and in neovessel formation in an aortic ring model [397]. In the current study, we attempted to define the role of a plant-derived SEGRM compound A (CpdA) in angiogenesis. For this purpose, we analyzed the impact of CpdA on the biology and function of human umbilical vein endothelial cells (HUVEC) and on neovessel formation in an *ex vivo* aortic ring model, and we compared the results with a synthetic GC dexamethasone (Dex).

Our results indicate that CpdA, similarly to Dex, does not negatively affect HUVEC viability (Figure 40A) and does not change endothelial characteristics of HUVECs (Figure 41). However, in contrast to the GC, CpdA has an inhibitory effect on HUVEC proliferation (Figure 40B). Moreover, we did not observe inhibition (or promotion) of tube-like structures (TLS) formation with Dex or CpdA after 6h of treatment, although the GC-mediated TLS formation inhibition *in vitro* was previously described after longer treatment times (22-24h) [344]. Nevertheless, in line with previous reports [397] we confirmed Dex's angiostatic properties in the *ex vivo* aortic ring model, in which the number of neovessel outgrowths was significantly reduced compared to the untreated control (Figure 42). CpdA seemed to have no positive or negative effect on neovessel formation in this assay.

HUVECs express glucocorticoid receptors (GRs) as visible on the indirect immunofluorescence images (Figure 39A). Treatment with Dex clearly triggered GR translocation to the nucleus, but this action was not exhibited by CpdA. Previously, CpdA was shown to translocate GR into the nucleus of A549 cells, although with a slightly lower efficacy than Dex [268]. Furthermore, CpdA induced a nuclear translocation of GR in GR-transfected LNCaP cells, clearly less proficient than the GC fluocinolone acetonide [353], and a similar outcome was reported in fibroblast-like synoviocytes [269]. However, we recently reported, in concordance with results obtained in HUVECs, a strongly impaired CpdA-mediated GR nuclear translocation in colon cancer-derived CAFs [389], which suggests that the efficiency of CpdA-mediated GR translocation depends on a cellular context. Interestingly, Dex-regulated genomic actions of GR in HUVECs were rather subtle as compared to other cell models [268,269,389,450], showing a relatively weak GR transactivation, reflected by only a 2-fold upregulation of GILZ (Figure 39C) and only a partial transrepression manifested by a suppression of IL-1β but not RANTES or TNFα levels. CpdA, on the other hand, displayed stronger suppressive properties, as it downregulated all three proinflammatory molecules (Figure 39D-E), without triggering GR-transactivation, confirming its dissociated nature and its functionality despite a poor GR translocation (Figure 39C). These combined results raise the question whether CpdA's actions in HUVECs are solely GR-mediated. It was previously documented that CpdA, next to GR-binding, could also bind another nuclear

receptor family member, the androgen receptor (AR), which resulted in the inhibition of AR function in prostate cancer cells [353]. Interestingly, CpdA displayed GR-independent actions on NFκB's transcriptional activity in rheumatoid arthritis fibroblast-like synoviocytes. In GR-knocked down cells, CpdA not only inhibited NFκB p65's nuclear translocation and binding to DNA, but it also displayed a significant impact on TNFα-induced MAPK activation, with the latter effect discovered to be GR-independent. However, in order to obtain significant anti-inflammatory effects of CpdA, presence of the functional GR was still necessary [270]. Furthermore, CpdA impaired the maturation and activation of LPS-treated bone marrow-dendritic cells via suppression of expression of several cell-surface and pro-inflammatory molecules. Interestingly, these CpdA-derived effects were also present when GR was pharmacologically blocked or siRNA knocked down, indicating a GR-independent inactivation of the NFκB intracellular signaling cascade [271]. Moreover, in the human airways smooth muscle cells, CpdA was shown to inhibit expression of several GC-resistant chemokines, in a GR-independent manner [273]. In our study Dex's effects on GR-transactivation and transrepression in HUVECs were relatively weak, thus, CpdA's additional anti-inflammatory mechanism via direct inhibition of NFκB might explain the more pronounced downregulation of IL-1β, RANTES and TNFα, since all three molecules' expression is regulated via an NFκB-responsive element in their promoters [451-453].

In this presented work, distinct angiostatic effects were observed in the *ex vivo* aortic ring assay, in which Dex inhibited neovessel outgrowths. Since this was not apparent with CpdA treatment it could be concluded that these effects were due to GR-transactivation or non-genomic events. However, in such a complex model as an aortic ring, it is important to account for indirect effects of the treatment via inflammatory cells, pericytes and other components present in the aortic tissue. Alternatively, CpdA is more labile than Dex and could be degraded, explaining lack of activity at longer time frames [454]. Macrophage-mediated inflammatory mechanisms occurring in the aorta in response to injury are required in neovessel formation [415]. In fact, inflammation is an important regulator of angiogenesis and the interconnections of these two processes is increasingly recognized not only in wound healing but also in cancer progression [455]. Inadequate oxygen supply (hypoxia) is a common denominator for both inflammation and angiogenesis and regulation of hypoxia involves recruitment of transcription factors common for inflammation and angiogenic responses, namely NFκB and hypoxia-inducible factor (HIF)-1. Interestingly, NFκB regulates expression of HIF-1 and vice versa [456]. And indeed, it is proven that blocking NFκB activity in prostate cancer cells causes inhibition of angiogenesis [457]. Surprisingly, targeted activation of NFκB in ECs seems to have an angiostatic outcome and it is investigated as a potential treatment strategy [458].

In conclusion, we showed that the SEGRM CpdA does not share the same angiostatic properties as Dex, but its anti-inflammatory properties are present and strongly pronounced in the HUVEC model. Besides its mild growth-inhibitory properties, CpdA seems to have little impact on HUVEC biology, and furthermore, it does not affect neovessel formation in an aortic ring model. Nevertheless, the impact of both GCs and SEGRMs ought to be researched in further detail, in order to identify the exact mechanism of action in ECs and in more complex models of angiogenesis.

PART IV: GENERAL DISCUSSION

1. Main findings and their significance

The role of glucocorticoids (GCs) in suppressing inflammation and fighting hematological malignancies is widely recognized [195]. Although GCs are also administered to patients with solid tumors, the influence of this treatment on cancer biology is not fully understood and often controversial [278,281]. The aim of this doctoral dissertation was to gain a more detailed insight into the role of GC treatment in colon cancer. Specifically, the objective of this project was to define the impact of GCs on cancer-associated fibroblasts (CAFs), and the effects of GC treatment via these CAFs on other cell populations within the cancer microenvironment, namely endothelial cells (ECs) and glucocorticoid receptor (GR)-deficient colon cancer cells. Moreover, we have compared direct effects of GCs and a selective GR modulator (SEGRM) compound A (CpdA) on the biology of CAFs, endothelial cells, and on angiogenesis as a functional read-out.

1.1. Glucocorticoids and CpdA differentially affect the biology of colon cancer-derived CAFs and human umbilical vein endothelial cells (HUVECs).

In Articles 1 and 4 we focused on direct effects of classic GCs, mainly dexamethasone (Dex), and the SEGRM CpdA, on the biology of colon cancer-derived CAFs and human umbilical vein ECs (HUVEC). Although the compounds exerted a number of similar effects in both cell lines, there were some marked exceptions noted as well. Neither compound negatively affected cell viability or typical myofibroblastic and endothelial markers. However, in contrast to Dex, CpdA displayed cytostatic effects in CAFs and in HUVECs. Growth-inhibitory effects of CpdA were previously described in malignant hematological cells, as well as in prostate and bladder cancer cells [351,353,446], and moreover, a GR-independent induction of apoptosis was reported in non-malignant cells, such as thymocytes and mouse embryonic fibroblasts [454]. Furthermore, Dex was very effective in triggering a GR translocation to the nucleus, where it positively affected GILZ mRNA expression in both investigated cell lines. In CAFs, Dex negatively affected the GR gene and subsequent protein expression, which are relevant to GC-induced downregulation of its receptor, known as a homologous downregulation, and are correlated with the occurrence of GC resistance [211]. CpdA did not trigger GR downregulation, therefore, it is less likely to evoke therapy resistance, as previously shown in fibroblast-like synoviocytes [352]. Moreover, CpdA was recently reported to have GR-independent therapeutic effects in GC-resistant conditions [273]. Nevertheless, a combined treatment of CpdA and Dex did not prevent GR downregulation in colon cancer-derived CAFs (Article 1, Figure 20C1).

In both cell lines, CpdA only marginally impacted GR translocation, and as expected, it did not show any GR transactivating properties [268]. Both compounds effectively suppressed a TNFα-

induced expression of several pro-inflammatory molecules. In CAFs, Dex proved to be more effective than CpdA by suppressing 5 out of 6 tested genes: RANTES, ICAM, MCP-1, IL-1β and TNFα, while CpdA suppressed only RANTES and ICAM expression. In contrast, in HUVECs CpdA exerted stronger anti-inflammatory effects than Dex, by suppressing all 3 tested genes IL1β, RANTES and TNFα, whereas Dex had suppressive effects only in the case of IL1β. The differences between Dex's and CpdA's transrepressive profiles among the cell lines and particular genes suggest cell-specific mechanism, and could potentially be caused, in view of CpdA's impaired GR translocation, by CpdA's GR-independent actions on the activity of NFκB in an yet unknown mechanism [270,271,441].

In conclusion, we confirm that CpdA behaves differently from classical GCs, does not trigger GR homologous downregulation, but has anti-inflammatory properties, which are most probably partially GR-independent, suggesting its potential role in cases of GC resistance. CpdA's role in delaying CAF and EC growth might have an additional therapeutic benefit in cancer progression.

1.2. GC treatment strongly affects the composition of the CAF-derived secretome.

In CAFs, Dex but not CpdA showed strong suppressive effects on the expression of HGF/SF, TGFβ and TNC, important secretory factors involved in cancer progression and/or angiogenesis. Further investigation (described in the Articles 2 & 3) revealed additional factors present in the CAF secretome that were sensitive to GC treatment. uPA, ANGPTL2, MMP-2 and prostaglandins levels were diminished in the conditioned medium obtained from Dex-treated CAFs (CM^{DEX}). Moreover, the potential enzymatic activity of MMP-2 and MMP-9 was also diminished. Interestingly, both mRNA and protein levels of ANG were increased due to Dex treatment (Table 9; Article 3, Table 8).

Cancer-protective and cancer-promoting properties of CAFs are gaining more attention in order to understand the intercellular cross-talk within the tumor microenvironment and to improve the applied therapeutic strategies. Recent studies focus on targeting not only the cancer cells but also CAFs in order to neutralize the cancer cell growth stimulatory effects of stroma. In pancreatic cancer nab-paclitaxel in combination with gemcitabine actually decreased CAF content and collagen architecture in patients, contributing to a more promising treatment outcome [437]. Other ongoing studies focus on an anti-TGFβ therapy and on blocking the cross-talk between cancer cells and stromal cells, along with an inhibition of angiogenesis [153,459,460].

In the Articles 2 & 3 we showed that the dramatic changes in the composition of the CAF-derived secretome caused by the GC treatment hold a potential to alter the behavior of other cellular populations within the tumor microenvironment.

Table 9. GC-sensitive factors detected in the CAF-derived culture medium and their role in cancer progression and/or angiogenesis.

Factor	Role in cancer and/or angiogenesis	Reference
Angiogenin	Stimulation of angiogenesis by influencing endothelial and smooth-muscle cells migration, invasion and proliferation. Induction of tube formation by ECs. Stimulation of tumor growth.	[105,433]
ANGPTL2	Stimulation of sprouting angiogenesis and EC migration. Positive regulation of endothelial colony forming cell vascular lumen formation. Association with chronic inflammation.	[409,461]
HGF/SF	Mitogenic, motogenic, morphogenic stimulation of epithelial and endothelial cells. Induction of cell invasion, tumorigenesis and angiogenesis. Anti-apoptotic properties in epithelial cells. Contribution to resistance against RAF inhibitors.	[134,462]
MMP-2, -9	ECM degradation, facilitating cell invasion and EMT, proteolytical activation of TGFβ.	[463]
prostaglandins	Stimulation of cancer cell proliferation and invasion. Induction of immunosuppression. Promotion of EC survival, migration and tube formation	[406,464]
TGFβ	Dual role as a tumor-suppressing and a tumor-promoting factor. Growth inhibition of early cancer cells. Immunosuppression, induction of EMT. Recruitment of CAFs. Pro- or anti-angiogenic functions depending on a cellular context.	[34]
tenascin C	Facilitating cell invasion, migration and EMT.	[77,465]
uPA	ECM degradation and remodeling, facilitating cell migration, invasion, cell extravasation and angiogenesis. Contribution to cell proliferation.	[110]

1.3. CAF-derived factors promote endothelial cell proliferation and migration, however, these pro-migratory effects are gone due to Dex treatment.

In Article 2 we focused on the role of CAF-derived conditioned medium (CM^{CTRL}) and CM^{DEX} on the biology of ECs, using mainly HUVEC as a model cell line. We showed that CAF-derived culture medium induced growth and migration of HUVECs, without affecting endothelial characteristics of the cells, confirming CAFs' stimulatory role in angiogenesis, previously described in several reports [139,140,385]. Although the levels of angiogenin, a factor that contributes to cell motility, were elevated after Dex treatment, we showed that in the secretome from GC-treated CAFs (CM^{DEX}) the pro-migratory properties were absent, however, the growth-stimulatory effects were preserved. We conclude that GC-mediated effects influenced the activity and/or levels of a pro-migratory factor(s) and/or inhibitors produced by CAFs. These data contribute to our understanding of GCs' angiostatic properties through their indirect effects on ECs via CAFs.

1.4. An indirect influence on endothelial cells via effects on CAFs and immune cells contributes to the angiostatic properties of GCs.

Although the angiostatic properties of GCs are widely recognized, the mechanisms behind them are not fully understood. Our results suggest that these effects could be caused by an indirect impact exerted on neighboring cells. We speculate that GC-mediated changes in the expression of multiple pro-angiogenic factors produced by neighboring cells strongly alter the behavior of ECs. The *in vitro* results presented in Article 4 showed that a direct application of Dex did not significantly affect EC behavior. This corresponds with previous reports, where a detailed investigation revealed that the angiostatic impact of GCs does not affect EC proliferation or migration but impairs *in vitro* tube formation [344]. Although we have not detected an inhibition of tube formation in HUVECs or HAoECs possibly due to use of a less precise method of screening, in the *ex vivo* assay we observed that Dex inhibited neovessel outgrowth formation, which corresponds with the *in vitro* process of tube formation and is in accordance with previous reports [397]. Nevertheless, it must be taken into account that the aortic explants comprise not only ECs but also other types of cells, of which the immune cells are known to play a key role in triggering neovessel formation [415]. Similar observations were made using CAF-derived conditioned medium (as described in Article 2). Although in the *in vitro* experiments CM^{CTRL} (or CM^{DEX}) had no significant effect on tube formation, the neovessel pro-growth effect of CM^{CTRL} was tremendous in the *ex vivo* model. These observations suggest that the impact of CM^{CTRL}, such as stimulation of IL-6 expression in ECs, also affected the activity of immune cells present in the vascular tissue. It is plausible that a direct GC application to the explant (also as a residue in CM^{DEX}) led to immunosuppression, and as such impaired the pro-inflammatory signaling exerted by immune cells, and indirectly inhibited neovessel formation.

1.5. Dexamethasone treatment neutralized promoting effects of CAFs on colon cancer cells.

In Article 3 we showed that GC treatment diminishes the cancer-promoting properties of CAFs. The stimulation of cancer cell growth and invasive potential via the treatment with CM^{DEX} was significantly less pronounced as compared to the stimulation with CM^{CTRL}. The results suggest GCs as potential agents to target the stroma-cancer interactions by blunting pro-aggressive signals released by CAFs. Many previous works reported on an increase of stroma activity due to radio- and chemotherapy and induction of therapy resistance. CAFs were shown to release elevated levels of IL-17 upon chemotherapy, which in turn promoted growth of colon cancer stem cells [162]. Moreover, CAFs were reported to affect the sensitivity to oxaliplatin and 5-FU in colorectal cancer cells [164]. Similar protective effects exerted by CAFs were described in other cases including pancreatic cancer [466], melanoma [163], head and neck squamous cell carcinoma

[165]. Therefore, our results suggest that a GC-mediated decrease in the activity of stroma, by impairing protective properties of CAFs, could also be beneficial during radio- and chemotherapy.

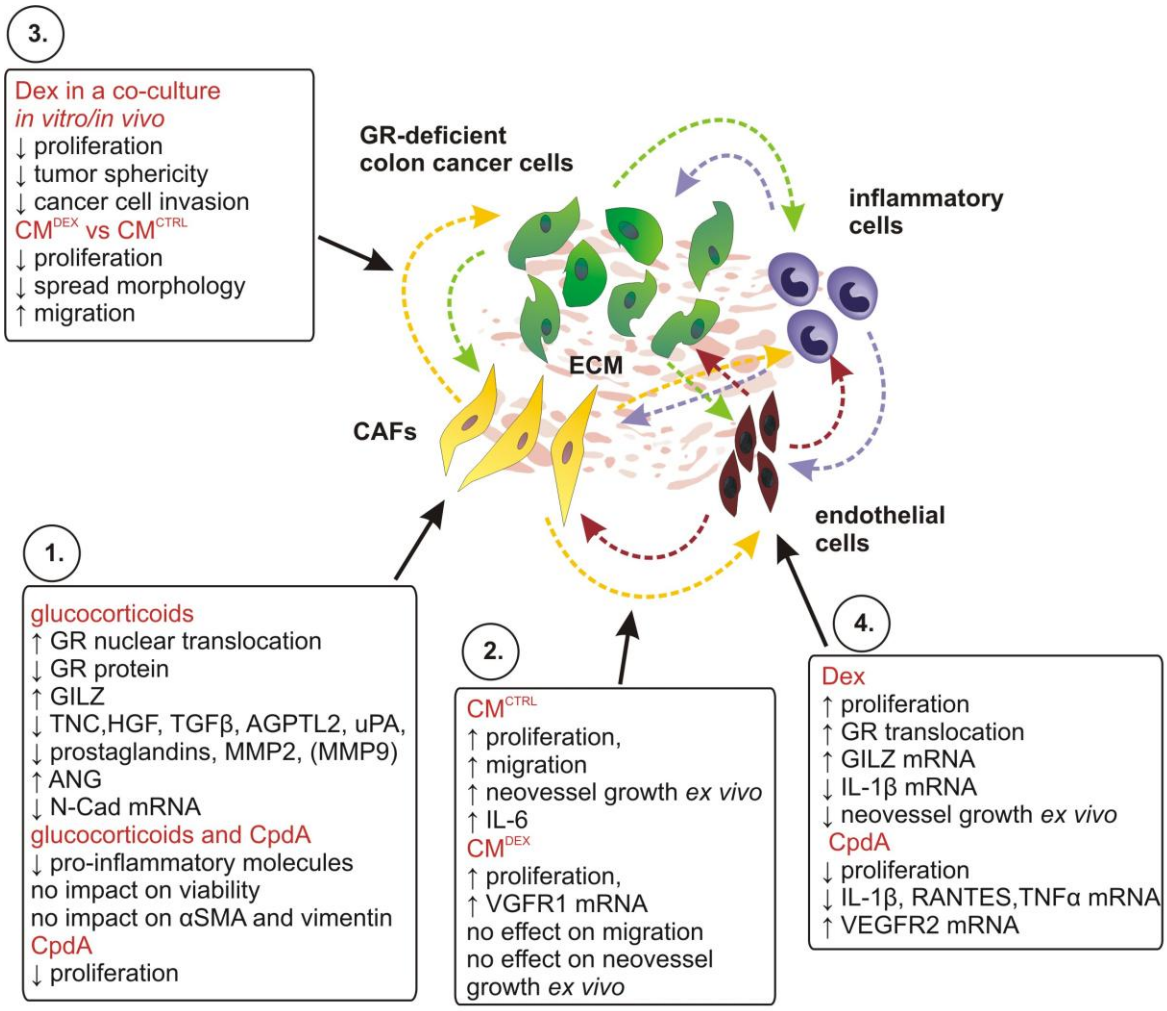

Figure 43. A schematic representation of cross-talk in the tumor microenvironment and the main findings described in this dissertation. Dotted arrows represent impact of particular cellular components on the neighboring cell populations; numbers 1-4 correspond with the chapter numbers in the Results section covering the addressed findings.

1.6. Dex affects tumor expansion in the *in vivo* co-culture.

In the Article 3 we investigated whether the *in vitro* results of experiments with use of conditioned medium correspond to a situation where cells are in a physical contact with each other. Consequently, we used an *in vivo* chick chorioallantoic membrane (CAM) assay which we optimized for our purpose. CAM is an example of a model that is more complex than the *in vitro* experiments. Owing to the presence of fibroblasts, vasculature and immune cells, it can mimic a tumor microenvironment. Moreover, CAM allows measuring several different parameters, such as growth, invasion, angiogenesis and metastasis [467]. In our model, cancer cells were seeded on top of the CAM together with CAFs and the developed tumors were observed after 5 days of incubation. The difference in tumor shape (sphericity) and cancer cell infiltration between Dex-

and solvent-treated tumors was very pronounced. Obtained results confirm our initial conclusion that treatment with GCs affects tumor growth and invasiveness. The presence of other types of cells in CAM tissue did not abolish the initial *in vitro* observation of a CAF-derived CM impact on cancer cells. On the contrary, the effects of Dex were even clearer than in the *in vitro* morphology assay, suggesting additional GC-mediated suppressive effects also occur via other chick tissues. This optimized model might serve in the future as a fast screening of GC-mediated changes in altered conditions, such as a co-treatment with chemotherapeutics (which is further elaborated on in the Future perspectives section).

In conclusion, results of our study support the idea of targeting the stroma as part of the anti-cancer therapy. Owing to their genetic stability, lower proliferation rate and lower tendency to mutate, CAFs are more favorable targets than cancer cells. Moreover, cancer-stroma interactions are most probably universal across different cancer types, therefore targeting the cross-talk between these cell populations forms a promising strategy in cancer treatment [167].

The main results obtained during this doctoral project are summarized and depicted in Figure 43.

2. Relevance of the study

GCs are used against inflammatory afflictions [236]. Moreover, GCs are applied in solid tumor therapy owing to their anti-edemic and anti-emetic effects, reduction of cancer-associated pain and ability to reduce post-surgical inflammation [281]. Nevertheless, a prolonged high dosage of exogenous GCs is associated with the occurrence of adverse effects [231]. Furthermore, several recent works reported detrimental effects of GCs on chemotherapy, stating that the direct GC treatment of cancer cells protects them from cytotoxic effects via inhibiting apoptosis [280,299]. In this light, the effectiveness and safety of GC administration during solid tumor therapy becomes questionable. Certainly, the answer whether GC treatment should be limited is dependent on various factors, such as cancer type and GR expression status of the cancer cells.

In this thesis we showed that attacking the CAFs with Dex limited cancer proliferation, invasion, and angiogenesis. Therefore, we suggest that GCs can have a beneficial outcome in cancer treatment via effects on stromal cells. Nevertheless, targeting only one cellular population with GCs without affecting other tissues, is virtually impossible. Interestingly, recent works focused on targeted drug administration and proposed an alternative solution for GC delivery via long-circulating liposomes (LCLs) [468]. Targeted GCs delivery could improve intratumoral drug concentrations, thereby reducing the systemic impact of GCs and occurrence of side effects [469]. LCLs have been previously reported to accumulate in malignant sites, due to the incidence of an increased vessel permeability within the tumor, as compared to a healthy vasculature [470]. Liposome-encapsulated prednisolone phosphate was proven effective at relatively low concentrations in reducing tumor growth of melanoma and colon cancer mouse models. In contrast, the free prednisolone phosphate did not have this anti-tumor effects when applied at the same treatment schedule, even when its concentration was increased [471]. The use of LCL-encapsulated GCs could be promising in the treatment of cancer, however, in the GR-responsive cancer cells the beneficial effects of GCs cannot be predicted taking into account GCs' role in chemotherapy resistance. Therefore, we suggest taking into consideration the GR expression status of patients' cancer cells when planning the strategy of a therapy. We speculate that GR-deficient cancer cells would not establish a chemotherapy-resistant phenotype and that the GC-mediated effects would be limited to the cancer microenvironment. Our results imply that due to the suppression of the expression of multiple cancer progression-associated molecules, such as HGF, TNC, and TGFβ released by stromal cells, cancer aggressiveness and angiogenesis would be effectively impaired.

Angiogenesis has been a target in cancer therapy since decades [472]. Bevacizumab, a mAb against the VEGF-A, is a standard therapeutic agent given to patients with metastatic CRC, applied

together with FOLFIRI or FOLFOX regimens. Moreover, aflibercept and regorafenib, agents disturbing the VEGF signaling, are also recently used in the therapy of metastatic CRC [42,45,46]. Previous studies recognized the anti-angiogenic potential of GCs via suppression of VEGF and other pro-angiogenic molecules secreted by cancer cells, including prostate and brain cancer, renal cell carcinoma, and head and neck carcinoma [287,335-337,339]. In the present study we showed that GC's tumor-suppressing properties can also originate from the decrease of pro-angiogenic molecules secreted by stromal cells, resulting in a lack of pro-migratory effects on the ECs.

A large body of evidence shows that non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin and diclofenac are potent and cost-effective preventive agents for CRC [59,473]. NSAIDs' anti-inflammatory actions originate from blocking the prostaglandin synthesis, mainly via inhibition of cyclooxygenase (COX)-2. Nevertheless, the mechanism behind the chemopreventive properties of NSAIDs is not fully understood [58,63]. GCs in comparison to NSAIDs are much stronger suppressors of inflammation, and next to other mechanisms, they also contribute to inhibition of prostaglandin production via suppression of phospholipase A2 and COX-2 [407]. Prostaglandins are not only mediators of inflammation, but they are also associated with carcinogenesis and angiogenesis. Prostaglandin (PG)E_2, the most abundant prostanoid found in the epithelial cancers, stimulates cell motility, proliferation, and induces production of pro-angiogenic factors, such as VEGF [464,474]. Interestingly, our results confirmed GC-mediated inhibition of prostaglandin synthesis also in CAFs. Whether GCs similarly to NSAIDs hold chemopreventive properties is not clear. GC treatment was shown not to correlate with the risk of CRC in a population-based study [306]. Moreover, in an *in vivo* study in carcinogen-induced mice, aerosolized budesonide was shown to prevent the development of lung cancer [288]. Furthermore, recently published results of a randomized trial in patients with indeterminate lung nodules revealed that after 1 year of treatment with inhaled budesonide the average size of non-solid lung nodules was substantially decreased compared to the placebo, over a 5 year follow-up [475]. Another recent study analyzed chemopreventive properties of several commonly used drugs and supplements, including budesonide, aspirin and ascorbic acid. The study was performed on a mouse model mimicking conditions in smokers, and confirmed chemopreventive properties of budesonide in mouse lung, however, budesonide administration was linked with occurrence of parenchymatous degeneration of the liver in mice [476,477]. Nevertheless, use of budesonide as a chemopreventive agent in human lung cancer seems justified owing to its short plasma half-life and selective retention by airway tissue [478]. Furthermore, an oral budesonide formulation holds therapeutic benefit in several inflammatory gastrointestinal conditions. It was shown effective in the induction of remission of Crohn's disease and collagenous colitis and it is the best-

documented agent for treatment of microscopic colitis [479]. Nevertheless, its role in chemoprevention of CRC is not documented. In CRC, aspirin was shown to be efficient as a chemopreventive agent especially when used at high doses for more than 10 years, which was also inevitably related with gastro-intestinal complications [473]. Since the prolonged use of GCs is associated with multiple detrimental side effects, the GCs in their present form are also less suitable for testing as potential long-term chemopreventive agents in case of CRC.

Consequently, there is an ongoing search for modified GCs and other GR ligands (SEGRAMs) that would hold beneficial properties without triggering the harmful side effects. Although a mechanistic distinction is difficult to make, it is hypothesized that GR-transrepressive actions exert in general more favorable effects than GR-transactivation (further referred to as "transrepression hypothesis") in chronic disorders [255]. Several SEGRMs, such as AL-438, ZK-216348, Mapracorat and CpdA were proven to exert a limited to a non-existent GR-transactivation, while their anti-inflammatory features were maintained and the typical GCs-associated side effects were decreased [259,264,268,350]. Therefore, this direction of research may bring solace, especially for patients suffering from chronic inflammatory conditions. Interestingly, several SEGRMs were also tested in cancer. RU24858, 21-hydroxy-6,19-epoxyprogesterone (21OH-6,19OP), Avicin D and CpdA were reported to have potential direct cancer-modulating properties [438]. In fact, CpdA exerted beneficial anti-tumor effects on leukemia cell lines (CEM and K562) via upregulating pro-apoptotic genes. Moreover, CpdA caused growth and survival inhibition in prostate cancer cells and displayed beneficial anti-androgen properties, and inhibited bladder cancer growth, invasion and migration [351,353,446]. Interestingly, a recent study in triple negative breast cancer even showed that in contrast to Dex, CpdA did not evoke chemotherapy resistance [419].

Nevertheless, the transrepression hypothesis is also subject to some critical opinions. The most important argument is that transrepression is not solely responsible for the beneficial outcome of GCs. In fact, the transactivation mechanisms are also needed to exert GCs' anti-inflammatory properties [480]. Dual specificity phosphatase (DUSP)1, also known as MAP kinase phosphatase (MKP)-1, an enzyme upregulated via GR-transactivation was shown to decrease levels of pro-inflammatory factors (including IL-6, IL-1β, and TNFα) via interference with MAPK and subsequently with the activity of AP-1 and NFκB [481-483]. Moreover, the role of the highly GR-transactivated gene GILZ is also recognized in suppressing inflammation. GILZ was shown to prevent nuclear translocation of AP-1 and NFκB and also to disturb MAPK signaling cascade [234]. Furthermore, there are other GC-inducible genes reported to have anti-inflammatory properties, such as tristetraprolin, IκB and IL-10 [480]. Nevertheless, GR's conformation is sensitive to the structure of the ligand, and moreover, different GR modulators attract different co-factors to the

receptor, as such significantly altering the GR-mediated expression profile. Therefore, the research on the non-steroidal ligands continuously holds a lot of promise [248]. Consequently, the quest for new GR ligands with improved safety profiles, is certainly justified.

3. Limitation of the study

During the course of this project we faced several problems and limitations associated with various factors, briefly described in this section.

3.1. GC-mediated pleiotropic effects

One of the troublesome aspects is the fact that GR is widely expressed in almost every human tissue. Therefore, in clinic it is difficult to restrain the effects only to the desired target tissue (in practice of particular inflammatory afflictions, local GC use in form of aerosol sprays and topical ointments is applied). The use of a GR-deficient colorectal cell line helped to dissociate the direct from indirect effects of GCs and allowed to monitor effects only attributed to GR-responsive CAFs. Moreover, it is important to note that GC-mediated effects are pleiotropic. GR-modulation affects the expression of a multitude of genes and the amount of changes prevents us to analyze them in detail. Having detected many GC-sensitive factors in the CAF-derived culture medium, it is difficult to find the one that is responsible for the observed effect, which most probably comprises chains of events, in which many factors are involved. We attempted to identify the role of several particular factors, by different approaches, such as selective depleting or blocking in the CM^{CTRL} or by addition of recombinant factors to CM^{DEX}. Although literature evidence and our experiments suggested TNC and HGF as synergistic stimulators of HCT8/E11 invasion, the simultaneous addition of those recombinant factors to CM^{DEX} did not result in a full restoration of the pro-invasive potential characteristic for CM^{CTRL} (Addendum 3, Supplementary Figure 13), suggesting involvement of other, yet unidentified factors (activators and inhibitors) affected by GCs.

3.2. Absence and presence of GR in cancer cells *in vitro* and in clinic

One of the initial objectives of this study was to examine the direct role of GCs and CpdA on a colon cancer cell line. However, despite of a well-known general abundance of GR expression in tissues, we found it difficult to find a cell line that was overtly responsive to GC treatment, and at the same time was invasion-inducible by contact with CAF-derived CM. Among the 6 colon cancer cell lines examined, all of them displayed a very low mRNA expression of GR (Figure 44A) and a weak or absent GILZ transactivation (Figure 44B), compared to the CT5.3hTERT CAFs and to the prostate cancer cell line PC3, which are highly responsive to GCs. The GR mRNA levels of colon cancer cell lines were comparable to a well-known GR-negative prostate cancer cell line LNCaP. Among the colon cancer cell lines, HCT116, despite showing low mRNA levels, displayed some protein expression and mild GILZ upregulation. Moreover, HCT116 was also susceptible to GC-induced GR homologous downregulation (Figure 45), making this cell line a potential model for future experiments. In the preliminary study, however, we did not observe a declined pro-invasive

potential of CM^{DEX} on this cell line (Figure 45B), highlighting a significant difference between the behavior of GR-positive (GR+) and GR-negative (GR-) cell lines. As previously reported, 50% of the colorectal tumor specimens from patients were GR-, thus, the use of the GR- cell line HCT8/E11 is relevant. Nevertheless being able to investigate the effects on GR+ cells would highly complement the general picture.

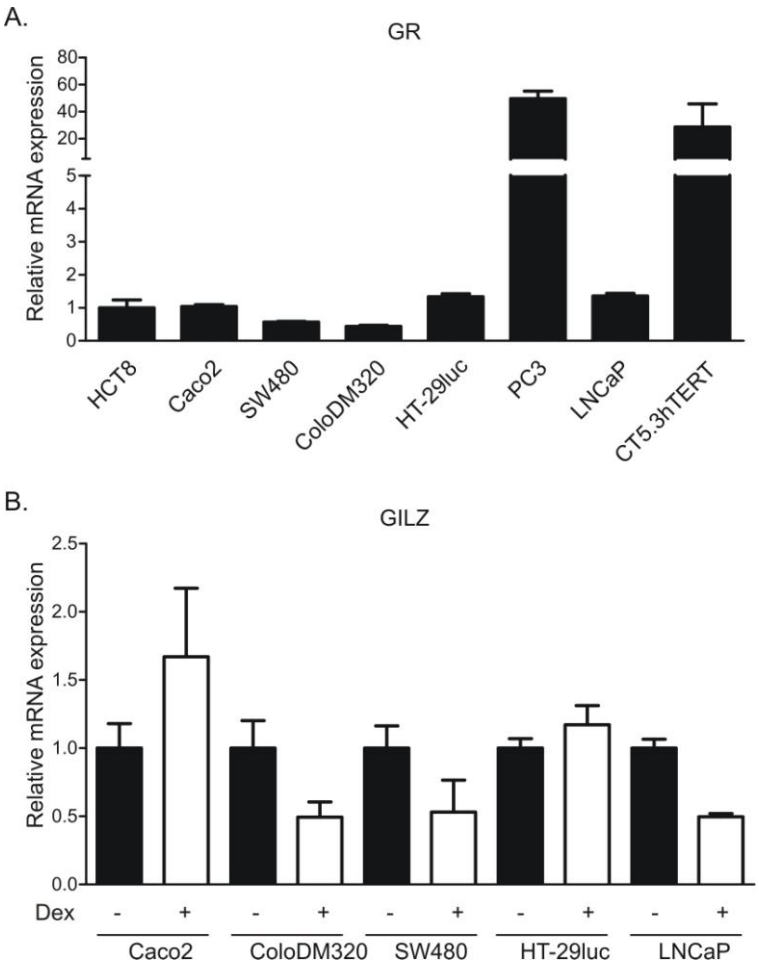

Figure 44. GR and GILZ mRNA expression in selected cell lines. Most of the widely available colon cancer cell lines showed very low GR mRNA levels compared to a well-known GR+ cell line PC3 and colon cancer-derived CAFs CT5.3hTERT. As expected, these colon cancer cell lines displayed a marginal to no GILZ upregulation after 6h Dex (1μM) treatment. As a negative control we used a prostate cancer cell line LNCaP-FGC, which is a well-documented GR- cell line.

3.3. Chemical instability of CpdA

Compound A, although used widely in research, was shown to convert into other derivatives, depending on the buffer system used for its dissolution. In the phosphate-buffered solutions CpdA decomposed into aziridine derivatives, reactive alkylating molecules with cytostatic properties [484]. In fact, CpdA evoked apoptosis in various cell types in a GR-independent manner. Further analysis of a dissolved CpdA showed that in pure water it decays into acetyl synephrine with a half-life ($t_{1/2}$) of approximately 5 days, which next transforms into synephrine

($t_{1/2}$ = weeks-months). CpdA stability in PBS is dependent on the pH. The $t_{1/2}$ increased inversely proportional to the pH value, ranging from approximately 3 to 100 minutes [454]. In order to ensure CpdA stability, the compound requires dissolving in absolute ethanol or DMSO in presence of the nitrogen vapors and further storage in -80°C. Although the protective conditions cannot be guaranteed in the cell culture or *in vivo* conditions, CpdA was suggested to be biologically active *in vivo* via stabilization by binding to plasma proteins [484]. Nevertheless, CpdA in its present form is not suitable for use in the clinic [454]. Although in our study CpdA did not cause cell death in CAFs and HUVECs, as shown via the viability assays, we observed a significant growth delay compared to a solvent control. We cannot exclude that these effects may derive from the CpdA's metabolites. Importantly, there are several other SEGRMs, which are proven more suitable for clinical application. The SEGRM Mapracorat (BOL-303242-X) is currently undergone clinical trials for treatment of allergic conjunctivitis, atopic dermatitis, and against inflammation following the eye surgery.

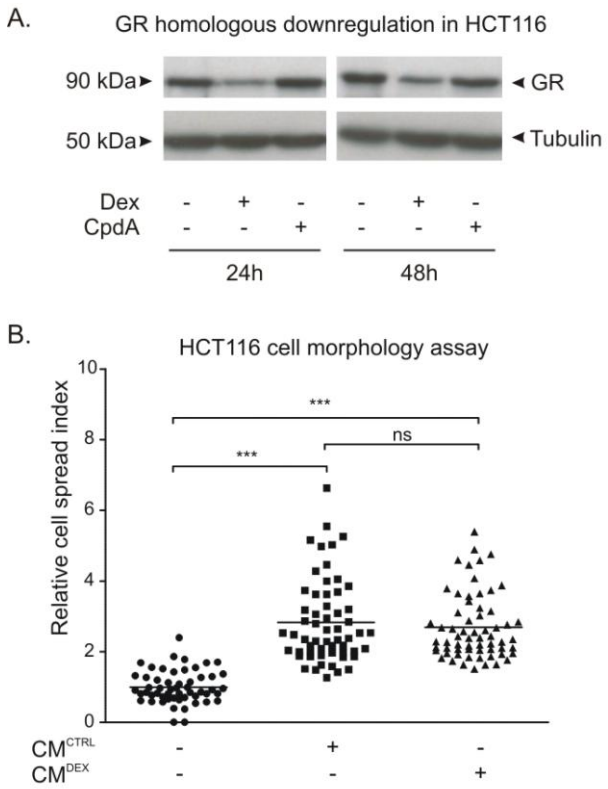

Figure 45. HCT116 are GR-responsive but do not react to CM^{DEX} in a similar manner as GR-deficient HCT8/E11 cells. A) After 24h and 48h of Dex (1μM) treatment GR levels have clearly diminished. B) The morphology assay showed that CM^{CTRL} increased spread morphotype of HCT116 cells, however, CM^{DEX} did not display diminished pro-invasive potential in these cells.

3.4. Homologous downregulation and GC resistance

Although in our *in vitro* study we observed clear GC-induced GR homologous downregulation in CAFs (Article 1, Figure 20C1), we did not observe any effects of GC resistance in the *in vivo* CAM model. It is likely that a longer treatment would result in a GC resistance and possible subsequent reversion to an invasive tumor phenotype, however, the CAM model does not allow a long-term incubation due to eventual egg hatching. Consequently, we attempted to use a xenograft mouse model to investigate the long term effects of the treatment, however, we have encountered unexpected technical problems which are described further.

3.5. The origin of the endothelial cells

HUVECs are widely used as human EC model, owing to their relatively easy and non-invasive means of isolation [485], compared to other human ECs. However, their behavior might vary from the tumor-derived ECs, due to their different origin. In the *in vitro* tube-like structure formation assay we also tested ECs from an aortic origin, HAoECs (Article 2, Figure 30). Although we did not observe any differences in responses to the CM between venous and arterial-derived cells, it would be useful to examine the behavior of HAoECs also in other experiments.

3.6. Use of the non-human experimental models

Experiments performed on non-human tissues always raise questions about the inter-species differences, which can be a cause of limited tissue response due to, for example, unmatching human-derived ligand and host-derived receptor [486]. In our study we used murine aortic tissue to investigate pro-angiogenic effects of human cell line-derived CM^{CTRL} and CM^{DEX}, however, we can extrapolate these results to the human situation only with a limited certainty. The same question emerged for the CAM assay. Although we observed interesting differences between Dex-treated and non-treated tumors, we must take into account that avian tissues and immune responses might substantially differ from the human situation.

3.7. *In vivo* studies in mouse model

In the course of this study we attempted to establish an *in vivo* mouse model using the in house engineered luciferase positive (HCT8/E11-luc-cl16) cells and the *In Vivo* Imaging System (IVIS) [422]. To this end, we subcutaneously injected female Swiss nu/nu mice with HCT8/E11-luc-cl16 cells together with CAFs, followed by injections of the experimental group with dexamethasone (15µg) 3 times per week, while the control group was injected with solvent (PBS). Contrary to our expectations, in both groups tumors did not progress. Moreover, in the control group all tumors shrunk and disappeared after 3 weeks, while in the experimental group most of the animals did not display any cancer cell-derived bioluminescent signal after 7 weeks (Figure 46). Although, the

animals did not develop the tumors as expected, we still observed a difference between the 2 groups. Treatment with Dex resulted in a significant delay of a tumor regression, revealing potential tumor-protective properties, probably owing to its immunosuppressive properties in the already immunocompromised mice. Therefore, it is important to repeat this experiment with the maternal HCT8/E11 cell line or with a more stable clone (as further described in the Future perspectives section).

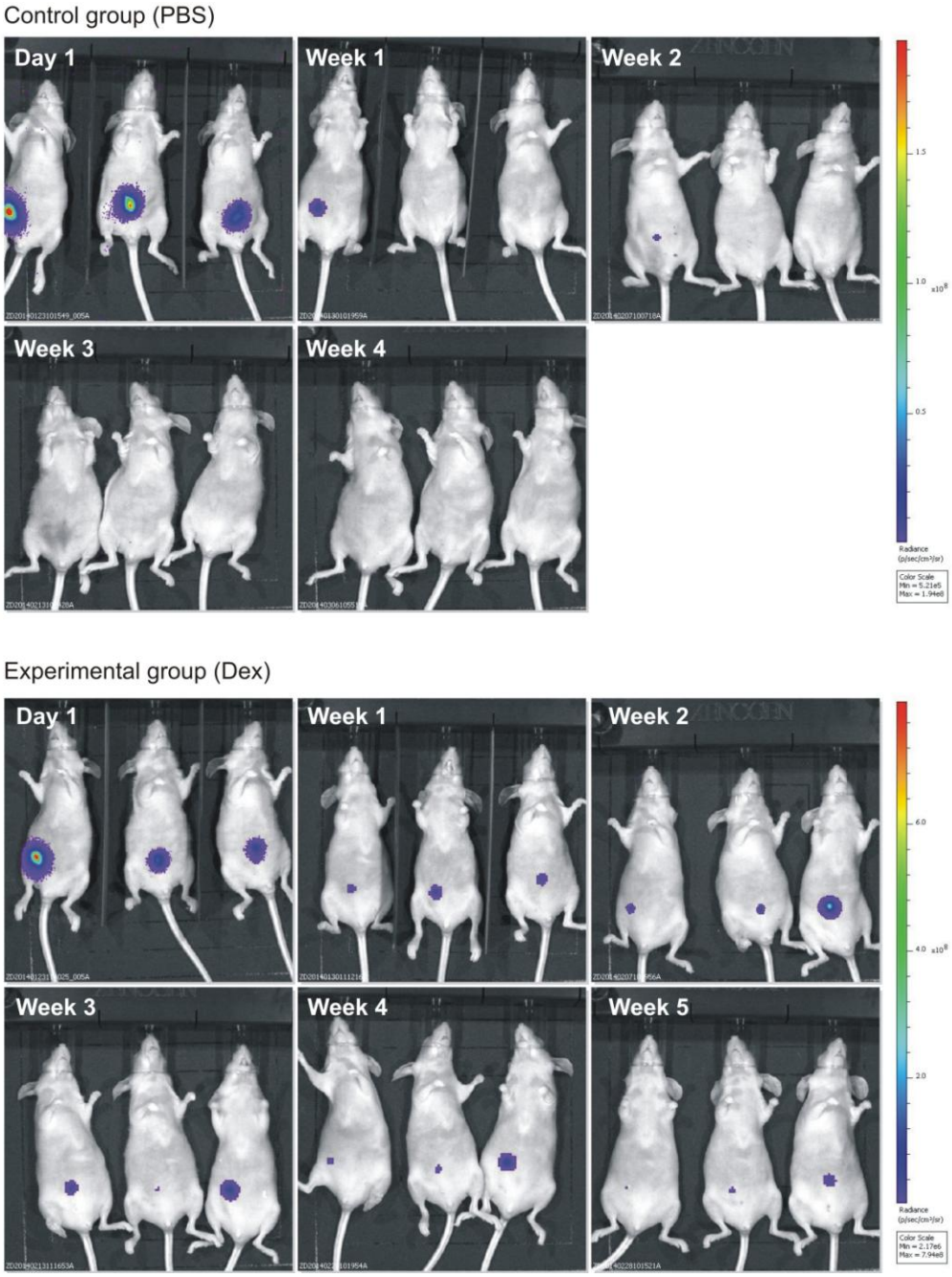

Figure 46. HCT8/E11-luc-cl16 cells did not develop tumors in mice. Nude Swiss nu/nu mice were injected with HCT8/E11 cells in mixture with CT5.3hTERT CAFs. Animals were injected with 15µg Dex (in PBS) or equivalent volume of PBS (control group). After three weeks, cancer cells were not detected in the control group. In the experimental group cancer cells were detected until week 7. Each picture represents triplet of the same animals over weeks per group.

4. Future perspectives

Although this study brought novel insights, it also raised several new questions. Finding the answers to these questions could improve our understanding on the role of GCs in the colon cancer microenvironment and the impact of CpdA.

4.1. The quest for new hits

In this study we presented several target molecules that are sensitive to GC treatment and are potential candidates for explaining the observed diminished cancer growth and invasiveness and lack of migration acceleration of ECs. Although the protein array performed on CAF-derived CM^{CTRL} and CM^{DEX} has already revealed or excluded potential candidates out of 507 available hits, a more detailed study would be necessary in order to find even more candidates. Mass spectrometry (MS) is a technique that helps to quantitatively identify molecules within a sample by ionizing them and sorting accordingly to their mass-to-charge ratio. MS is widely used in pharmacokinetics and protein characterization [487]. Via MS we could reveal in an unbiased manner more of interesting molecules in the secretome of CAFs that are affected by GCs and may contribute to GC-mediated beneficial effects. Moreover, the use of quantitative proteomics could strongly enrich our knowledge on the differences between particular factors' levels in the CAF secretomes of different treatments [488].Nevertheless, the more potential factors involved, the more difficult to decipher the mechanism behind the beneficial roles of GC treatment. It is highly possible that multiple factors act together in a specific manner to result in a final outcome.

4.2. *In vivo* experiments in mice

With the use of our optimized CAM model we showed that the treatment with Dex diminishes tumor growth and invasion. It would be interesting to confirm these data on a long-term scale with use of animal models, such as Swiss nu/nu mice. Although our first attempt with the HCT8/E11-luc-cl16 failed, further experiments conducted by collaborators revealed another clone HCT8/E11-luc-cl04 that successfully established the tumor formation in the control animals. Alternatively, the parental cell line could be also used, as described previously, albeit the advantage of the luciferase system is an easy visualization [428].

4.3. Impact of GCs and CpdA on GR-responsive colon cancer cells

Another interesting aspect is a detailed analysis of a response to direct and indirect GC/SEGRM treatment by the GR+ cancer cells. As mentioned before, it was not straightforward to find a highly responsive GR+ colon cancer cell line. An alternative approach is to work with stably transfected GR+ colon cancer cells or ideally, with primary cultures obtained from patients. GR+ cells should be analyzed in terms of survival, proliferation and invasion after direct treatment with

GCs and CpdA, but also with CM^{CTRL} and CM^{DEX}. Furthermore, an RNA-sequencing approach, combined with MS for functional protein read-out, in such GR+ cancer cell lines would shed more light on the role of target genes of inflammatory mediators, factors involved in EMT, cell invasion and angiogenesis in the GR+ cancer cells after such treatment. Further *in vivo* experiments could confirm how GR-responsive cancer cells applied together with the CAFs react to Dex and CpdA treatment.

4.4. Effects of chemo- and radiotherapy in combination with GCs on CAF secretome

It was previously shown that ionizing radiation (IR) activates tumor stroma, making it more supportive for cancer cells [159]. It would be interesting to investigate whether the combination of GC administration and exposure to IR or chemotherapy changes the activity of CAFs, by analyzing the CM^{DEX+IR} and $CM^{DEX+CHEMO}$ expression profiles compared to the CM^{IR}, CM^{CHEMO} and CM^{CTRL}, and by examining its effects on the colon cancer cell proliferation, invasion and migration.

So far, there has been little information published about the impact of GCs in combination with chemo- or radiotherapy on fibroblasts in general. The GC-mediated protection against radiation and drug cytotoxicity was earlier investigated in various cancer cell lines [280]. GC-mediated protection against apoptosis was observed in glioma cells, however, the researched mechanism was most probably cell-type specific. Mouse embryonic fibroblasts (MEFs) did not profit from GC treatment since their survival was decreased due to exposure to a chemotherapeutic teniposide. However, in MEFs with knocked-out p21, a major target of p53 actions, the protective influence of Dex was re-established [489]. Interestingly, Brock and colleagues showed that Dex had no protective properties on human diploid fibroblasts AG1522 when exposed to ionizing radiation, in contrast to V-79 cells, fibroblasts obtained from Chinese hamster lung tissue [490]. In case of internal radiation therapy, a study performed on samples obtained from synovial tissue from rheumatoid arthritis patients showed that the combined local treatment with glucocorticoids and yttrium-90 did not affect the number of fibroblast-like synoviocytes compared with the GC treatment alone [491]. Both cortisol and the endogenous rodent GC corticosterone were found to reduce pro-inflammatory protein expression (including IL-6, IL-8, and GM-CSF) of X-irradiated human skin and lung fibroblasts [322].

In general combination of a chemotherapeutic agent with a GC has found application in the treatment of keloid and hypertrophic scars and in ocular lesions, resulting in synergized anti-proliferative effects and suppression of inflammation. An antineoplastic antibiotic mitomycin C (MMC) has been recently used for prevention of keloid and hypertrophic scar recurrence [492]. A conjugate of MMC with the GC triamcinolone was shown to effectively reduce NIH 3T3 fibroblasts growth, an effect which was similar to MMC treatment alone [493]. In a small clinical study

performed on patients with several keloid scars, topical application of MMC followed by injections with triamcinolone prevented recurrence of keloid scars to a similar extend as the treatment with GCs alone [494]. A slowly-released combination of the antimetabolite 5-FU with triamcinolone was investigated in subglottic stenosis, a narrowing of airway due to scarring. The drug combination reduced subglottic stenosis formation in rabbits, which was evidenced by suppressed inflammation and fibrosis formation compared with the control group of non-treated animals [495]. *In vitro* studies investigated the mechanisms behind the synergy between low-dose 5-FU and triamcinolone in reduction of keloid scars recurrence. Keloid fibroblasts' proliferation was stronger suppressed by a 5-FU-triamcinolone combination than by triamcinolone alone. Although, triamcinolone held no pro-apoptotic effects on the fibroblasts, induction of cell death with the drug combination was more effective than with 5-FU alone. Moreover, the combination treatment strongly inhibited collagen type I expression, and stimulated MMP-2 expression [319].

A combination of MMC and Dex showed interesting results on corneal fibroblasts, indicating this treatment regimen could be beneficial for corneal wound healing. Dex did not increase the pro-apoptotic properties of MMC, but it suppressed MMC-upregulated expression of IL-8 and MCP-1 at mRNA and protein levels and attenuated MMC-stimulated actions of p38 MAPK and Jun N-terminal kinases (JNK) [496]. Similarly, in human Tenon's capsule fibroblasts isolated from patients suffering from pterygium, Dex inhibited MMC-induced IL-8 upregulation, and moreover, it reversed MMC-triggered cell growth inhibition associated with elevated IL-8 levels. These results hold beneficial implication for postoperative recovery of the cornea [497]. 5-FU in combination with GCs has also been shown effective in treatment of ocular lesions. Conjugate of 5-FU with Dex, as well as suspension of 5-FU combined with triamcinolone applied intravitreal to rabbits with induced proliferative vitreoretinopathy resulted in regression of the disease compared to the untreated group [498].

A recent study investigated a link between MMC and circadian clock gene expression in NIH 3T3 fibroblasts and ocular fibroblasts. The circadian clock system is regulated by light, subsequent production of corticosteroids in the adrenal glands, and GR activity [499]. MMC not only decreased Dex-induced nuclear GR protein levels, but subsequently led to the inhibition of Dex-induced *Per1* transcription, a gene implicated in circadian rhythm regulation, and decreased SGK-1 expression, a well-known direct target of activated GR [500].

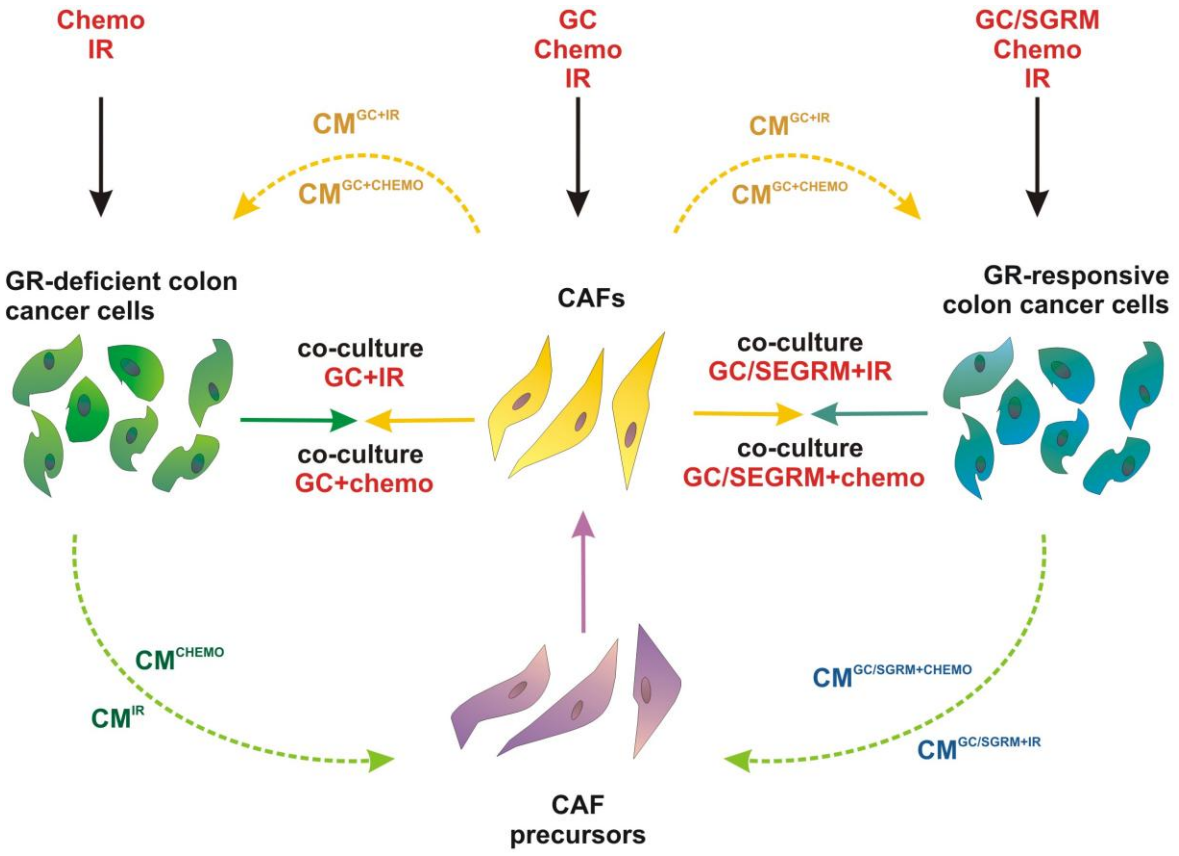

Figure 47. A schematic representation of proposed future experimental approaches. Different experimental set-ups can be designed based on the knowledge obtained during this doctoral project.

4.5. Effects of GC treatment via CAFs on radio- or chemotherapy-induced GR-positive and GR-negative colon cancer cells

As it was reported before in cell lines derived from various cancer types, including colon cancer, GC treatment is suspected to trigger chemotherapy resistance via anti-apoptotic actions [299,311]. In this perspective, another interesting question is how the GC-affected stromal cells influence chemotherapy resistance in GR+ and GR- colon cancer cell lines. Such experiments should be conducted using CAF-derived CM^{CTRL} and CM^{DEX}, and oxaliplatin- and Dex-treated colon cancer cells. But also in co-culture system using our optimized CAM model, where the tumors containing CAFs and colon cancer cells could be simultaneously treated with chemotherapeutic agent and Dex. Similar experiments with use of irradiated colon cancer cells would also shed light on the effects of GCs in combination with this kind of therapy.

4.6. The influence of GC-treated CAFs on tumor-associated macrophages

CAFs strongly impact the cancer microenvironment via secretion of various growth factors, enzymes, cytokines, chemokines, ECM proteins and adhesion molecules [76]. CAFs are implicated in several hallmarks of cancer including both tumor-promoting inflammation and avoiding immune destruction [8,127]. Cancer cells and CAFs were shown to attract tumor-promoting

myeloid-derived suppressor cells, including neutrophils, monocytes, immature dendritic cells, and early myeloid progenitors. Moreover, the increased CAFs' affinity to M2 macrophages in CRC has been associated with tumor-promoting effects, considering M2's role in immunosuppression via secretion of IL-10 and TGFβ and induction of angiogenesis [127,501,502]. In prostate cancer, CAFs were shown to attract monocytes to the tumor site and contribute to their transformation into M2 macrophages via stromal-derived growth factor-1 (SDF-1). Moreover, this impact was reciprocal, since the macrophages stimulated differentiation of fibroblasts into CAFs [503]. Among known immunomodulatory factors secreted by CAFs are IL-6, IL-8, RANTES, MCP-1, GM-CSF, and CXCL-1 [76,504]. Although via the protein array we detected only a part of these factors (Supplementary Table 2, Supplementary Figure 3, p.218), an mRNA analysis in the TNFα-induced CAFs revealed strong elevation of proinflammatory genes expression, including IL-6, MCP-1, ICAM, RANTES, IL-1β, and TNFα itself (Results Chapter 1, Figure 21, p.88). In the same experiment we showed that GC Dex strongly inhibited TNFα-stimulated expression of almost all of these molecules. These results suggest that an immunomodulatory role of CAFs is strongly impaired due to GC treatment. Suppression of such signals could not only decrease tumor-promoting inflammation but also possibly decrease macrophage infiltration, pointing to another beneficial side of an indirect GC impact. On the other hand, it is important to highlight that a direct GC treatment on lymphoid cells has been widely recognized, especially in hematological malignancies, owing to GC-mediated pro-apoptotic effects in lymphocytes [277]. The GC-mediated anti-proliferative actions were also reported in monocytic/macrophage cell lines [505]. Moreover, a strong immunosuppressive impact of GCs was recognized in macrophages and neutrophils, leading to inhibition of inflammatory mediator secretion including IL-1β and MCP-1 and subsequent reduction of myeloid cells infiltration into the tissue in a contact hypersensitivity model [506]. Combined, considering the little information published so far, a study analyzing the role of GC-treated CAFs on tumor-associated macrophages and vice versa would enrich our knowledge on an indirect GC impact in the tumor microenvironment and its role in tumor development.

4.7. The influence of GC-treated colon cancer cells on CAFs and their precursors

Cancer cells recruit CAFs by releasing factors, such as TGFβ and PDGF [35]. It would be important to find out whether GC/SEGRM treatment of GR+ colon cancer cells affect the production of these factors and, as such impact the transition of fibroblastic precursors into CAFs. Transition of the fibroblastic precursors, such as commercially available bone marrow-derived mesenchymal stem cells (BM-MSC), could be monitored during the treatment with conditioned medium from treated

and untreated GR+ cancer cells, by tracking the expression of myofibroblastic markers, such as αSMA and vimentin via RT-qPCR and Western Blot.

4.8. Continuation of the study on effects of Dex and CpdA on angiogenesis

In Article 4 we presented a study on direct effects of Dex and CpdA on the EC biology. These preliminary data revealed interesting properties of CpdA, which exerted anti-inflammatory and cytostatic effects in HUVEC. Moreover, we hypothesize that part of CpdA-derived effects in ECs are GR-independent. To this end, we would like to extend this research to other EC cell lines and to a detailed analysis of NFκB activity upon CpdA treatment, also in conditions with a knocked-down GR. It would be also useful to investigate other cell functions, such as migration and adhesion. New systems and technology including the xCELLigence and IncuCyte platforms allow us to investigate in an automated, more detailed and more efficient way the effects of treatment *in vitro*. Results from such *in vitro* systems will yield a better understanding of the fundamental effects in non-complex conditions and, as such provide a better starting set up for more complex conditions, such as use of CAM and mouse models.

Figure 47 depicts examples of potential future experimental set-ups based on the information gained from this doctoral project.

General conclusion

The present study demonstrated that a treatment with the GC Dex, via inducing severe changes in the composition of the CAF secretome, neutralized pro-invasive and growth-promoting impact on colon cancer cells. Moreover, Dex administration limited CAFs' pro-migratory properties exerted on endothelial cells, contributing to GC-mediated angiostatic effects. Our observations reveal interesting beneficiary properties of GCs, which upon further research, could be implemented in the strategy of colon cancer therapy.

Moreover, data collected on the properties of the SEGRM CpdA confirmed its anti-inflammatory properties and revealed new information about its functionality in CAFs and in ECs. Owing to their unquestionable potential, this project strongly supports further research on novel GR ligands.

ABBREVIATIONS

11β-HSD	11β-hydroxysteroid dehydrogenase
36B4 (RPLP0)	acidic ribosomal phosphoprotein P0
5-FU	5-fluorouracil
A	adenine
Ac-LDL	acetylated-low density lipoprotein
ACTH	adrenocorticotropic hormone
ADAM	a desintegrin and metalloproteinase
ADAMT	a desintegrin and metalloproteinase with thrombospondin motifs
AF	activation function
ALK	activin receptor-like kinase
Ang	angiogenin
ANGPT	angiopoietin
ANGPTL-2	angiopoietin-like 2
AP-1	activator protein 1
APC	adenomatous polyposis coli
AR	androgen receptor
ATP	adenosine triphosphate
AUC	area under curve
bFGF(R)	basic fibroblast growth factor (receptor)
BTM	basal transcriptional machinery
C	cytosine
CAF	cancer-associated fibroblast
CAM	chorioallantoic membrane
cAMP	cyclic adenosine monophosphate
CAT	collective to amoeboid transition
CBG	corticosteroid-bound globulins
CBP	CREB-binding protein
CCL5 (RANTES)	chemokine (C-C motif) ligand 5
CD	cluster of differentiation
CDK	cyclin-dependent kinases
CIMP	CpG island methylator phenotype
CIN	chromosomal instability
CM	conditioned medium
CM^{CTRL}	CM from solvent-treated cells
CM^{DEX}	CM from Dex-treated cells
COPD	chronic obstructive pulmonary disease
COX-2	cyclooxygenase-2
CpdA	compound A
CRC	colorectal cancer
CREB	cAMP response element-binding
CRH	corticotropin-releasing hormone
CRPC	castration-resistant prostate cancer
CSC	cancer stem cell
CTC	circulating tumor cell
CTLA	cytotoxic T-lymphocyte-associated antigen
CyP	cyclophilin
DBD	DNA-binding domain
Dex	dexamethasone
DMEM	Dulbecco's modified Eagle medium

DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
DUSP-1 (MKP-1)	dual-specificity phosphatase-1
EC	endothelial cell
E-cad	epithelial cadherin
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF(R)	epidermal growth factor (receptor)
EGM	endothelial growth medium
ELISA	enzyme-linked immunosorbent assay
EMDR	environment-mediated drug resistance
EMT	epithelial-to-mesenchyma transition
eNOS	endothelial nitric oxide synthase
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
FA	fluocinolone acetonide
FAP	familial adenomatous polyposis
FCS	fetal calf serum
G	guanine
GAG	glycosaminoglycan
GAP	GTPase activating protein
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC	glucocorticoid
GDP	guanosine diphosphate
GILZ	glucocorticoid-induced leucine zipper
GM-CSF	granulocyte-macrophage colony-stimulating factor
GR	glucocorticoid receptor
GRB2	growth factor receptor-bound protein 2
GRE	glucocorticoid-responsive elements
GSK3β	glycogen synthase kinase 3β
GTP	guanosine triphosphate
HAoEC	human aortic endothelial cell
HAT	histone acetyl transferase
Hcrt	hydrocortisone
HDAC	histone deacetylase
HGF	hepatocyte growth factor
HIF	hypoxia inducible factor
HNSCC	head and neck squamous cell carcinoma
HPA	hypothalamic-pituitary-adrenal
HRE	hypoxia-responsive elements
HRP	horseradish peroxidase
hsp	heat shock protein
HSPG	heparan sulfate proteoglycans
HUVEC	human umbilical vein endothelial cell
ICAM-1	intercellular adhesion molecule 1
IGF-1	insulin-like growth factor 1
IL	interleukin
IR	ionizing radiation
KRAS	Kirsten rat sarcoma
LBD	ligand-binding domain

LCC	latency competent cancer
LCL	long-circulating liposomes
LDH	lactate dehydrogenase
LDL	low density lipoprotein
LPS	lipopolysaccharide
luc	luciferase
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MAT	mesenchymal to amoeboid transition
MCP-1 (CCL2)	monocyte chemotactic protein 1
MDCT	multi detector computerized tomography
MDR	multidrug resistance
MEF	mouse embryonic fibroblasts
MKP-1 (DUSP-1)	mitogen-activated protein kinase phosphatase 1
MMC	mitomycin C
MMP	matrix metalloproteinase
MMR	mismatch repair
MR	mineralocorticoid receptor
mRNA	messenger RNA
MS	mass spectrometry
MSI	microsatellite instability
mTOR	mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N-cad	neural-cadherin
NCoR	nuclear receptor co-repressor
NES	nuclear export signal
NFκB	nuclear factor κB
nGRE	negative GRE
NK	natural killer
NKT	natural killer T cell
NL	nuclear localization
NLS	nuclear localization signals
NO	nitrix oxide
NPC	nuclear pore complex
NR	nuclear receptor
NSAID	non-steroidal anti-inflammatory drug
NTD	N-terminal transactivation domain
p53	protein 53
PAGE	polyacrylamide gel electrophoresis
PAI	plasminogen activator inhibitors
PARP	poly ADP-ribose polymerase
PBS	phosphate-buffered saline
PD-1	programmed cell death protein 1
PDGF	platelet-derived growth factor
PG	proteoglycan
PI3K	phosphoinositide-3 kinase
PIP_3	phosphatidylinositol-3,4,5-trisphosphate
PKB (Akt)	protein kinase B
PLC-γ	phospholipase C-γ
PlGF	placental growth factor
PPIB	peptidyl-prolyl cis-trans isomerase B
PR	progesterone receptor

Pred	prednisolone
PTM	post-translational modification
qPCR	quantitative polymerase chain reaction
RANTES (CCL5)	regulated on activation, normal T cell expressed and secreted
RNA	ribonucleic acid
ROS	reactive oxygene species
RT	reverse transcription
RTK	receptor-tyrosine kinase
RWD	relative wound density
SDF-1	stromal cell-derived factor 1
SDS	sodium dodecyl sulfate
SEGRA	selective glucocorticoid receptor agonist
SEGRM	selective glucocorticoid receptor modulator
SF	scatter factor
SGK-1	serum glucocorticoid regulated kinase
SH	steroid hormone
SLRP	small leucin-rich proteoglycan
SMRT	silencing mediator for retinoid or thyroid-hormone receptors
SRB	sulforhodamine B
SRC	steroid receptor coactivators
STAT	signal transducer and activator of transcription
SUMO	small ubiquitine-related modifier
T	thymine
$t_{1/2}$	half-life
TAM	tumor-associated macrophage
TGFβ	Transforming growth factor β
TH	$CD4^+$ helper T-cells
TIE	tyrosine kinase with immunoglobulin-like and EGF-like domains
TIMP	tissue inhibitor of proteinase
TLS	tube-like structure
TNC	tenascin C
TNFα	tumor necrosis factor alpha
TNM	Tumor Node Metastases
tPA	tissue plasminogen activator
Treg	CD4+ T regulatory cells
uPA	urokinase plasminogen activator
VEGF(R)	vascular endothelial growth factor (receptor)
ZEB	zinc finger E-box-binding homeobox
αSMA	alpha smooth muscle actin

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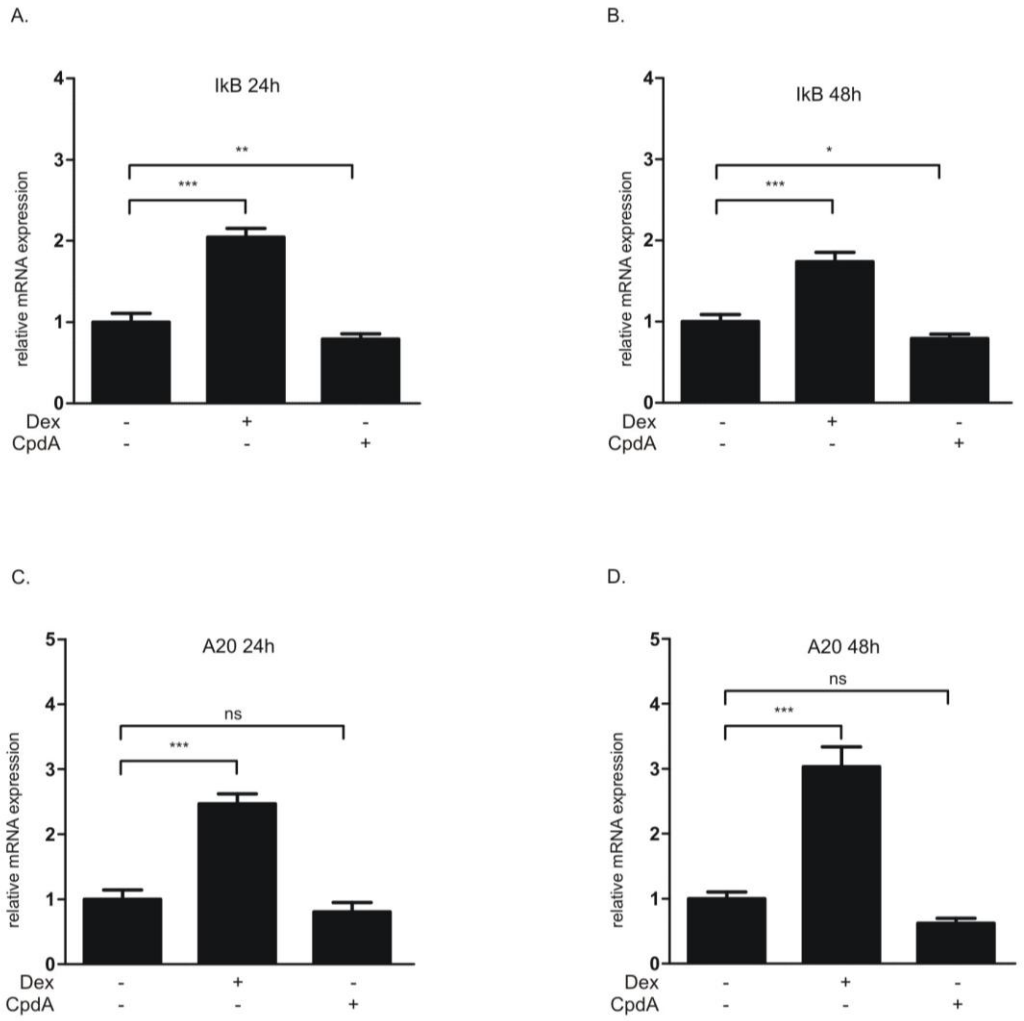
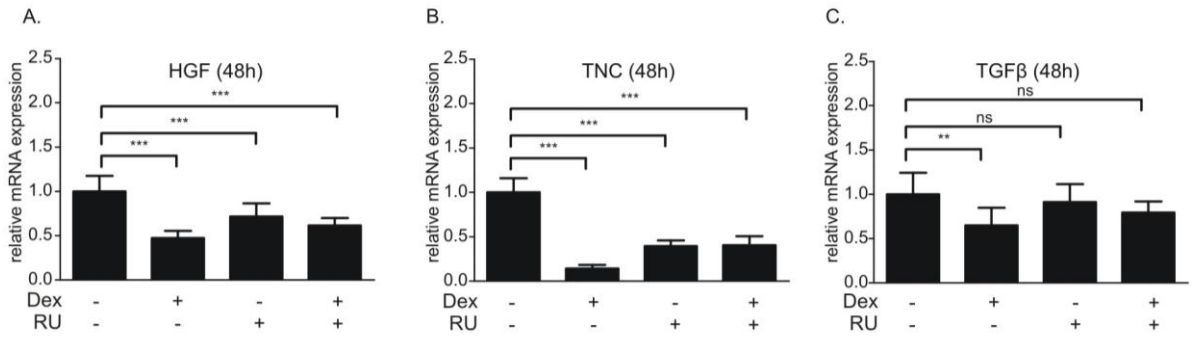
ADDENDUM 1.

Supplementary figures for Part III: Results: Chapter 1



Supplementary Figure 1. (A,B,C,D) CT5.3hTERT cells were treated with solvent, Dex (1µM) or CpdA (10µM) for 24h or 48h. Isolated mRNA was subjected to RT-qPCR assaying IκBα (A,B) or A20 (B,C) mRNA levels, and results were normalized to the respective geometric mean of GAPDH, PPIB and 36B4 household genes' mRNA levels. The solvent condition was set at 1 and results were recalculated accordingly. Results are the mean ±SD of two independent experiments and statistical analysis on potentially significant differences was performed using a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test. ns not significant,* p<0.05,** p<0.01, ***p<0.001.



Supplementary Figure 2. CT5.3hTERT cells were treated with solvent, Dex (1µM), RU (2µM) or co-treated with Dex (1µM) and RU (2µM) for 48h. Total mRNA was subjected to RT-qPCR assaying HGF (A) TNC (B) and TGFβ (C) mRNA levels, and results were normalized to the respective geometric mean of GAPDH, PPIB and 36B4 household genes' mRNA levels. The solvent condition was set at 1 and results were recalculated accordingly. Results are the mean ±SD of three independent experiments and statistical analysis on potentially significant differences was performed using a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test. ns not significant, **p<0.01, ***p<0.001.

ADDENDUM 2.

Supplementary data for Part III: Results: Chapter 2.

1. Supplementary methods

Acetylated-low density lipoprotein (Ac-LDL) uptake assay

HUVECs were seeded onto glass coverslips (coated with 0.1% gelatin) and incubated overnight at 37°C, 5% CO_2. Cells were then incubated for 24h in $EGM2^{S+}$ (control), DMEM, CM^{DEX} or CM^{DEX}. DMEM and 10-fold concentrated CM were diluted 1:1 with $EGM2^{S+}$. Ac-LDL uptake was assessed as described [449]. Briefly, HUVECs were incubated for 5h with 5 µg/ml Ac-LDL conjugated with alexa-488 (Invitrogen, Glasgow, UK, cat no: L23380), then washed with PBS, fixed with 2% paraformaldehyde, washed again with PBS and stained with DAPI. Images were taken using a fluorescence microscope (Axioscope, Zeiss, Oberkochen, Germany), CoolSNAP camera (Photometrics, AZ, USA) and MCID Basic 7.0 software. Photographs were analyzed for green fluorescence signal intensity over number of cells per image, using ImageJ software [362].

2. Supplementary tables

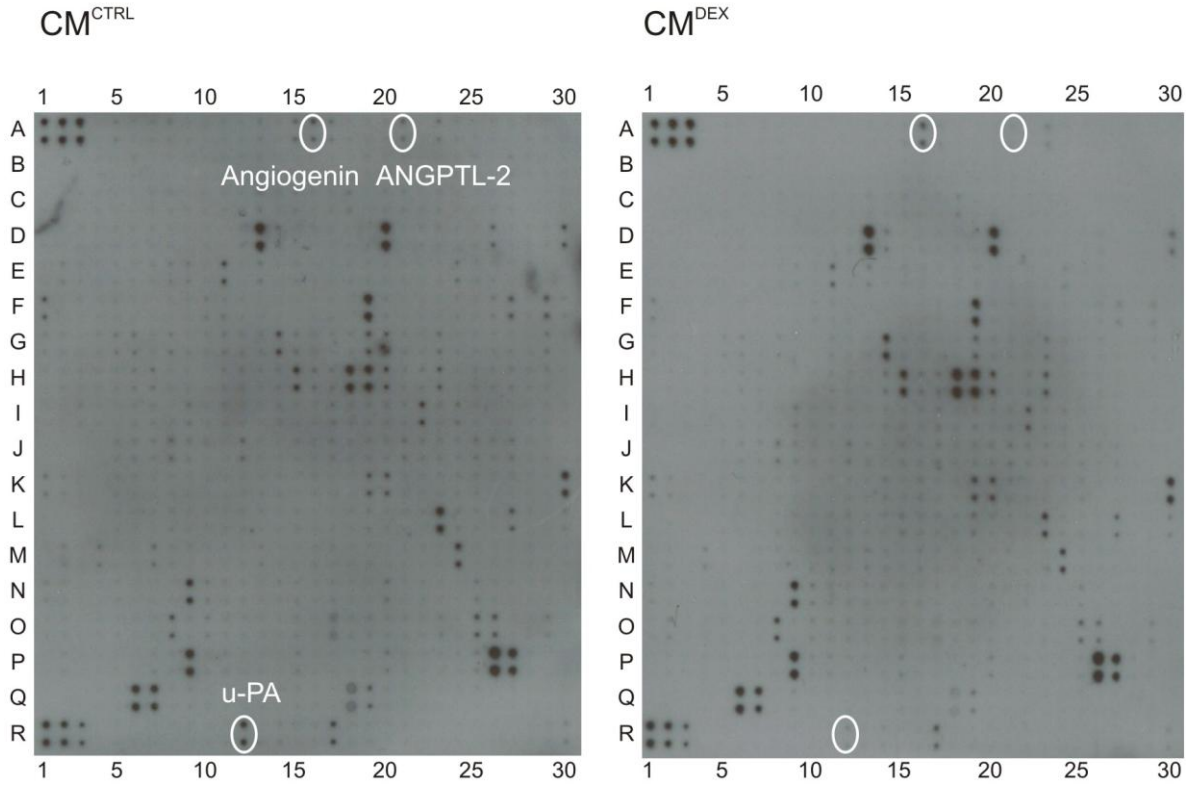
Supplementary Table 1. Conditioned medium dilutions and concentrations used in experiments

Figure	Cell Type	Experiment	Conditioned medium : EGM2	Conditioned medium final concentration	EGM2 used for dilutions
28A	HUVEC	MTT assay	1:1	5x	+serum (2%)
28B	HUVEC	SRB assay	1:1	5x	+serum (2%)
28C	HUVEC	SRB assay	1:1	5x	serum-free
29, suppl. fig. 7	HUVEC	scratch assay	1:1	5x	serum-free
30, suppl. fig. 8	HUVEC HAoEC	TLS formation	1:4	2x	+serum (2%)
31	HUVEC	RT-qPCR	1:1	5x	+serum (2%)
32, 33, suppl. fig 9.	murine aortic rings	aortic ring assay	1:1 with DMEM	5x	serum-free DMEM
suppl. fig. 4	HUVEC	ELISA	1:1	5x	+serum (2%)
suppl. fig. 5	HUVEC	Ac-LDL assay	1:1	5x	+serum (2%)

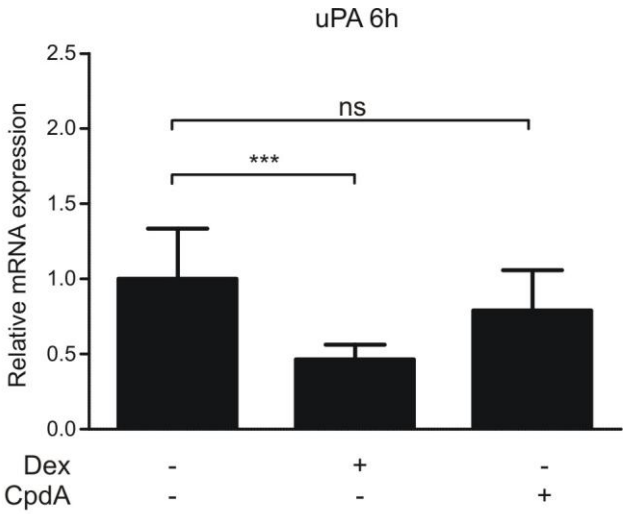
Supplementary Table 2. A summary of selected factors detected or not-detected in the conditioned medium from CAFs (position refers to location on the protein array in the Supplementary Figure 3; signal detection fold changes above the threshold 1.5 or below 0.66 are indicated in bold).

Factors detected in CM	position	CM^{DEX}/CM^{CTRL}	Factors detected neither in CM^{DEX} nor CM^{CTRL}	position
Angiogenin	A16	**2.24**	Angiopoietin-2	A18
Angiopoietin-1	A17	0.83	Angiopoietin-4	A19
Angiostatin	A23	1.09	ANGPTL-1	A20
ANGPTL-2	A21	**0.18**	bFGF	E16
Endostatin	D30	0.81	GM-CSF	G16
HGF/SF	G29	0.76	IL-1α	H24
IL-8	I24	0.91	IL-1β	H25
MCP-1	L23	0.88	IL-6	I20
TNFβ	Q18	0.73	PDGFs	N26-N30
TSP-1	P27	1.02	TNFα	Q17
u-PA	R12	**0.18**	TSP-4	P29
VEGF	R18	0.87	VEGFR-2	R18

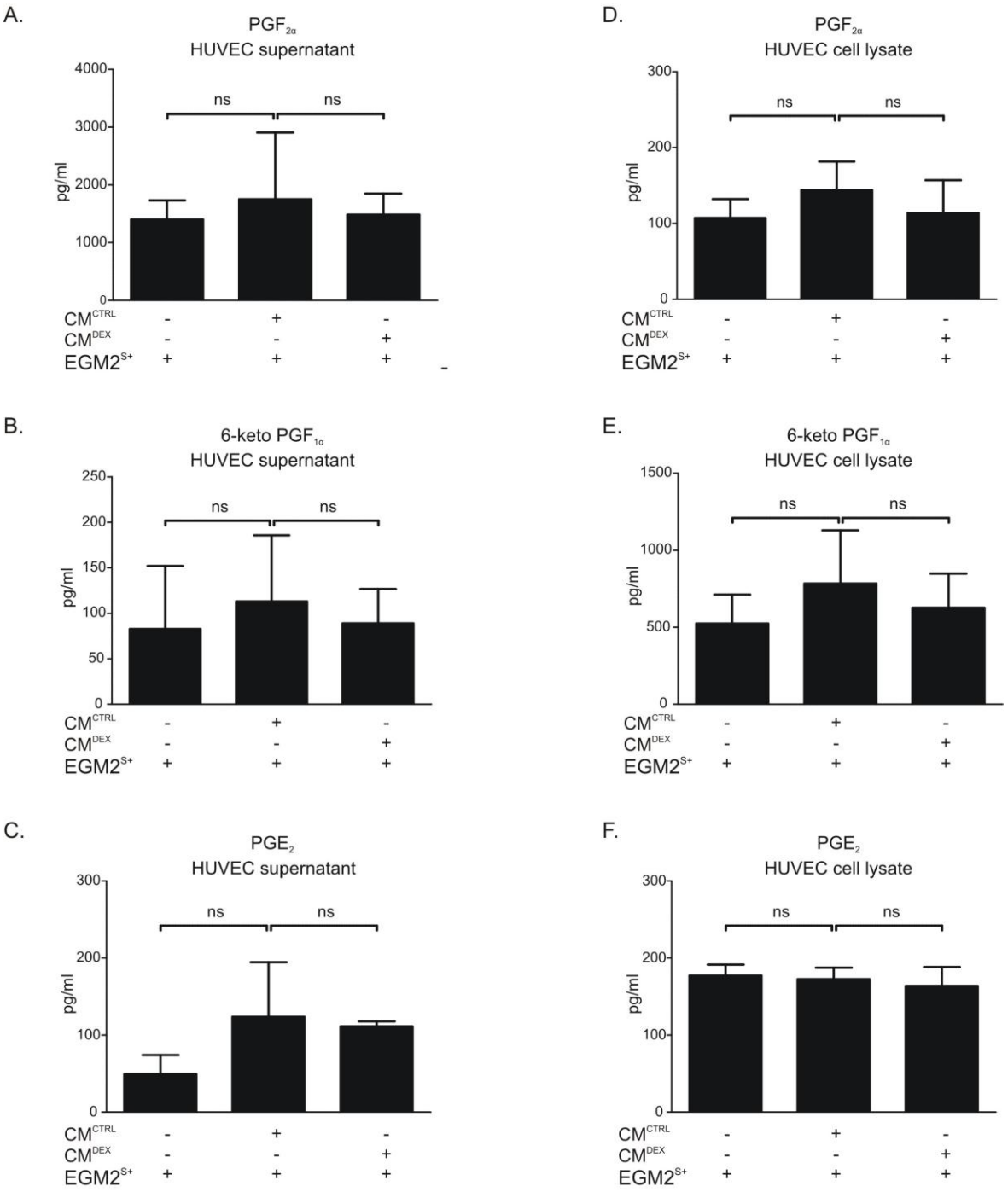
3. Supplementary figures



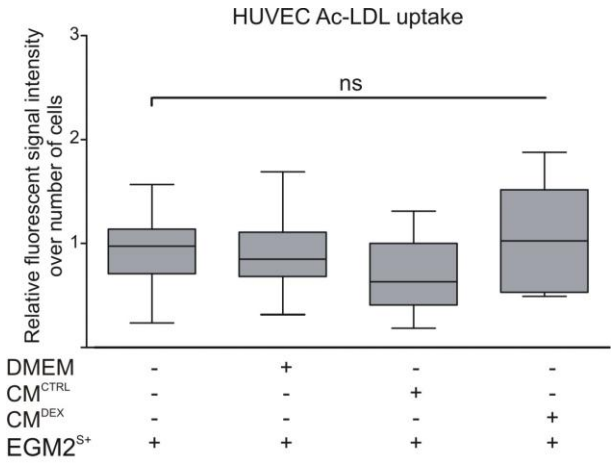
Supplementary Figure 3. Glucocorticoids alter the expression and secretion of proteins from cultured CAFs. CT5.3hTERT cells were treated with solvent or Dex (1μM). After 48h cell supernatants (CM^{CTRL} and CM^{DEX}, respectively) were collected, 4-fold concentrated and subjected to Ray Bio® Biotin Label-based Human Antibody Array I. Factors with a fold change above 1.5 or below 0.66 are indicated with white circles. Positions of selected factors are listed in table Supplementary Table 2. Positive controls are represented by dots in positions A1-3 and R1-3.



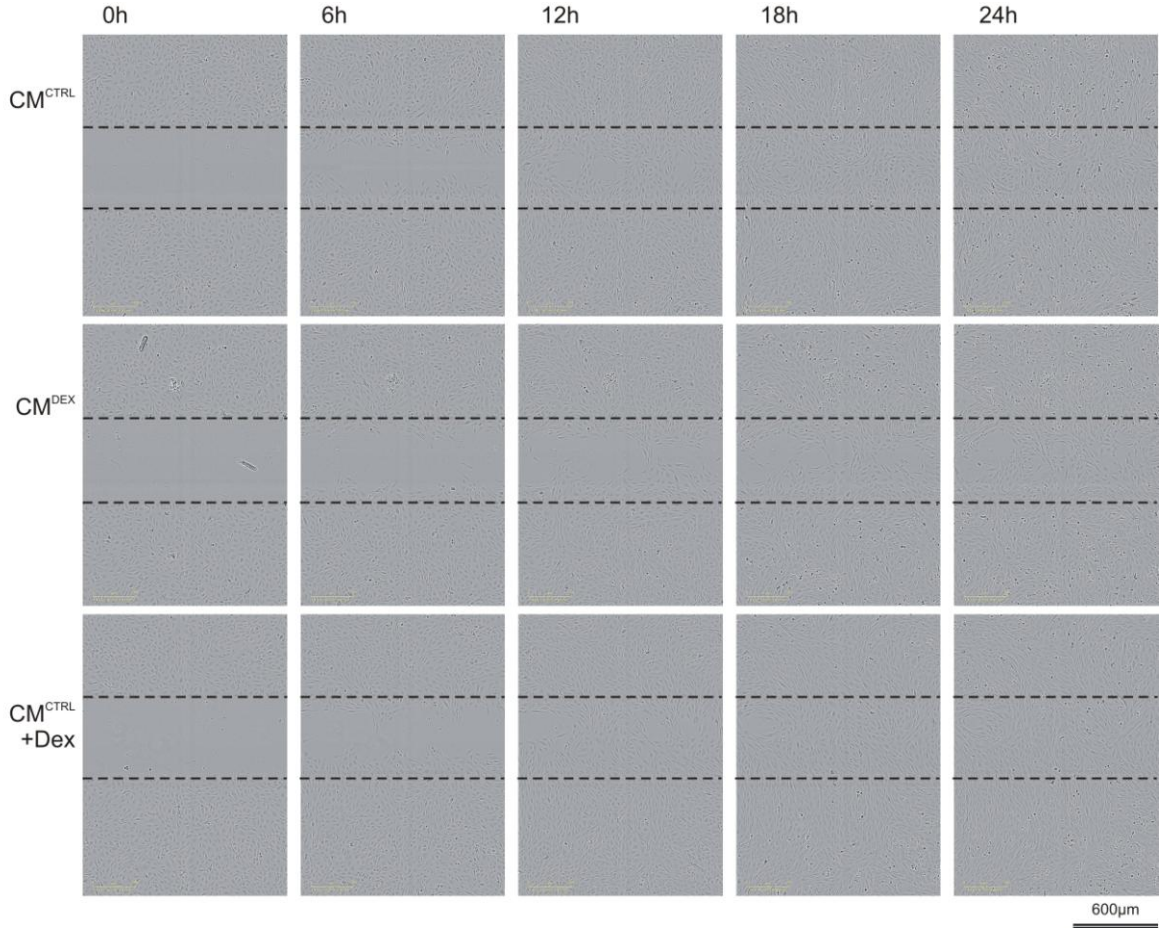
Supplementary Figure 4. Expression of uPA in conditioned medium is reduced when the CAFs are exposed for 6h to dexamethasone. CT5.3hTERT cells cultured in DMEM were treated for 6h with solvent, Dex (1µM) or CpdA (10µM). Isolated mRNA was subjected to RT-qPCR assaying uPA mRNA levels. Results were normalized to the respective geometric mean of GAPDH, PPIB and 36B4 reference genes' mRNA levels and are expressed relative to the solvent control. Results are the mean ± SD of three independent experiments and statistical analysis was performed using a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test. ns: not significant ***: $p<0.001$.



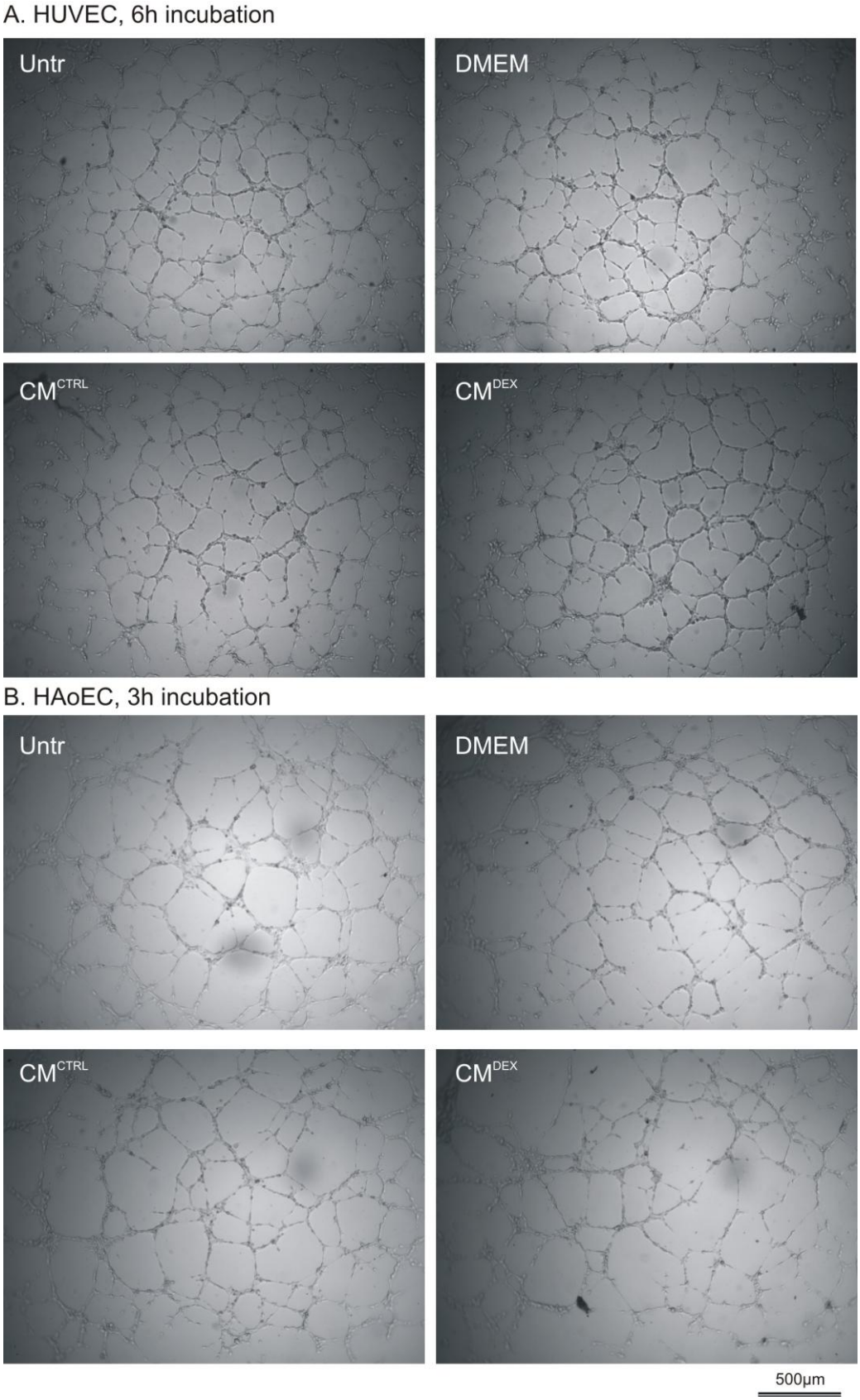
Supplementary Figure 5. Levels of prostanoids produced in HUVECs are not affected by treatment with conditioned medium from CAFs. (A-F) HUVECs were treated with either $EGM2^{S+}$ or $EGM2^{S+}$ mixtures with CM^{CTRL} or CM^{DEX} in 1:1 ratio. After 24h cells and media were collected and analyzed for prostanoids. In HUVEC media and cell lysates ELISA was performed to quantify (A, D) $PGF_{2\alpha}$, (B, E) PGI_2 (by assessing 6-keto-$PGF_{1\alpha}$) and (C, E) PGE_2 concentrations. Results are the mean ± SD of three independent experiments and statistical analysis was performed using a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test, ns: not significant.



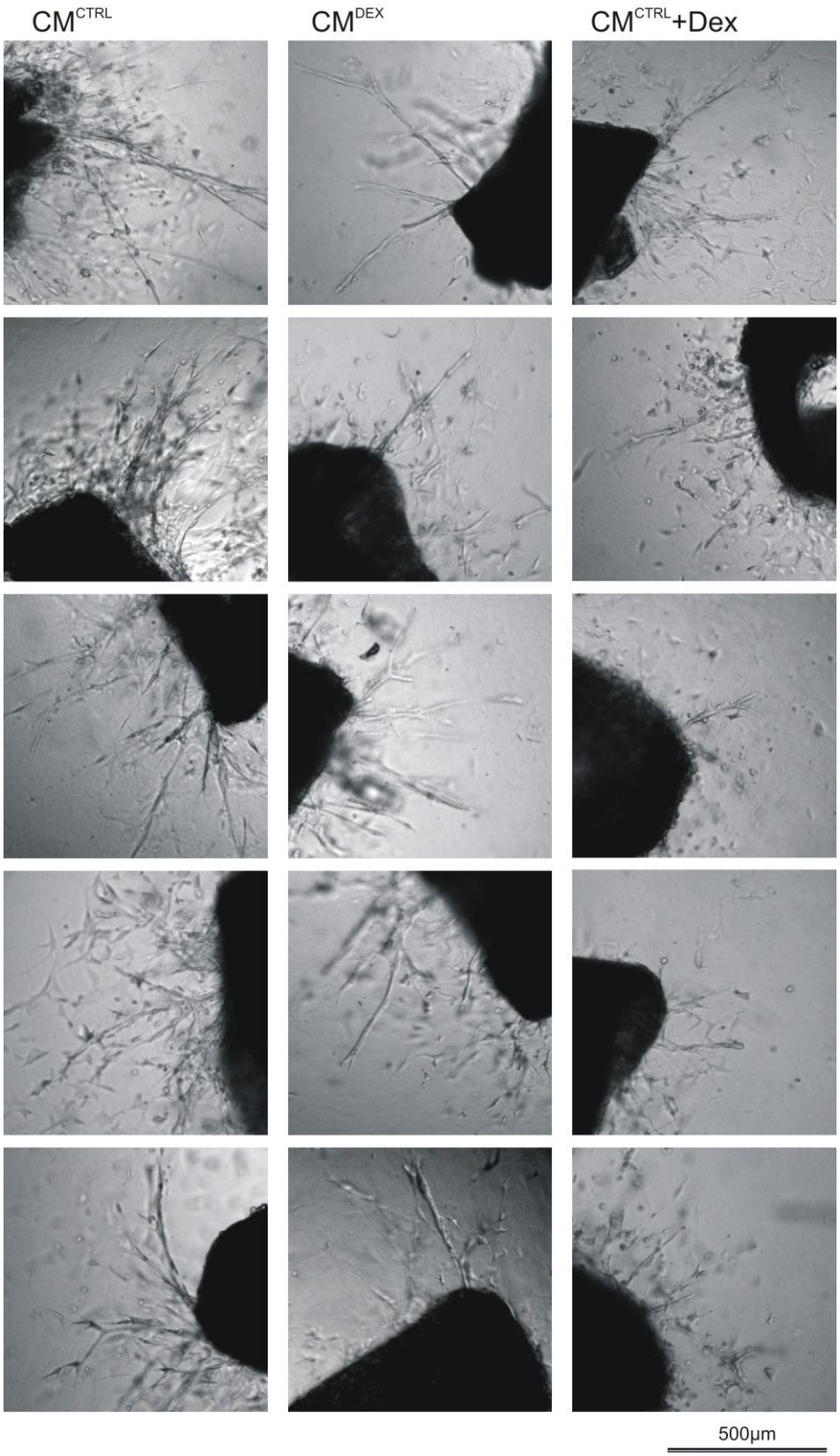
Supplementary Figure 6. Conditioned media does not alter acetylated LDL uptake by HUVECs. HUVECs were seeded onto coverslips and cultured for 24h in either EGM2^{S+} (control) or with EGM2^{S+} containing DMEM, CM^{CTRL} or CM^{DEX} in 1:1 ratio. Ac-LDL conjugated with Alexa-488 (5 µg/ml) was added to the cells (5h) before they were washed, fixed, counterstained (DAPI) and quantified. Tukey's box plots represent data of three independent experiments and were analyzed using a Mann-Whitney U test. ns: not significant.



Supplementary Figure 7. Representative images of scratch wound assay. HUVECs were cultured in EGM2^{S+}. After 18h a wound was created in the confluent cell monolayer. Cells were washed and treated with EGM2^{S-} mixtures with CM^{CTRL}, CM^{DEX} or CM^{CTRL}+Dex (50 nM) in 1:1 ratio. Examination of the wound healing process and image capture were performed with the IncuCyte ZOOM system. Dotted lines indicate the position of the original scratch wound.



Supplementary Figure 8. Representative images of tube-like structure formation assay. HUVECs (A) and HAoECs (B) were seeded on Matrigel-coated wells and treated with either $EGM2^{S+}$ or $EGM2^{S+}$ mixtures with DMEM, CM^{CTRL} or CM^{DEX} in 1:4 ratio. Phase-contrast images were taken at 6h post induction for HUVECs and 3h post induction for HAoECs.



Supplementary Figure 9. Representative higher-power images of the aortic rings and outgrowths.Explants were prepared from aortas isolated from adult male C57BL/6 mice. After embedding in collagen, aortic rings were treated with CM^{CTRL}, CM^{DEX} or CM^{CTRL}+Dex (50nM), in 1:1 ratio with serum-free DMEM. Images of explants and vascular sprouts were captured after 10 days.

ADDENDUM 3.

Supplementary data for Part III: Results: Chapter 3

1. Supplementary methods

Cell viability and metabolic activity (MTT) assay

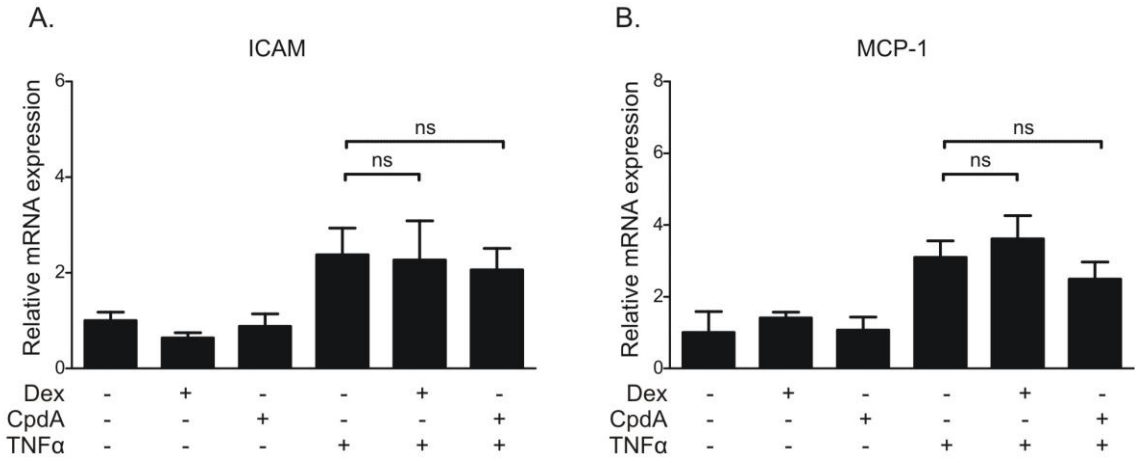
To test the cell metabolic activity and viability, HCT8/E11 cells were seeded in 96-well plates ($7.5x10^3$/well). In order to exclude treatment's effect on cell proliferation, we used confluent cell cultures, which were subsequently treated for 72h with CM^{CTRL} or CM^{DEX}, or with serum-free DMEM. Additionally, as a negative control, we included cells treated with 10% triton for 1h (data not shown). To analyze cell metabolic activity a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed, as described [360]. Plates were scanned using a ParadigmTM Detection Platform (Beckman Coulter®, Krefeld, Germany) and SoftMax® Pro 6.1 software.

2. Supplementary table

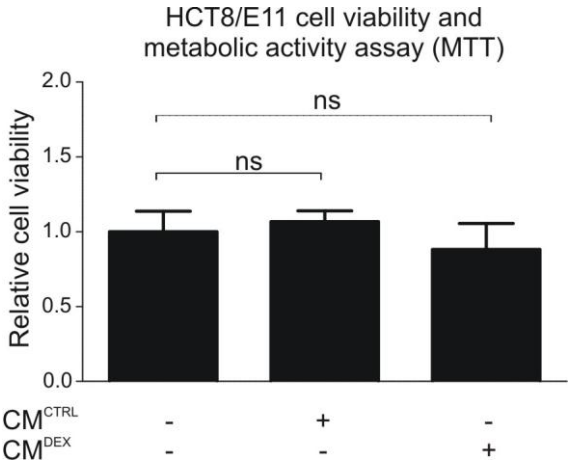
Supplementary Table 3. Scoring system used to evaluate CAM results.

Score	Tumor shape (sphericity)	Cancer cell infiltration
1	Semi-transparent, flat layer of cells	No cancer cells invading the mesenchymal layer, unchanged chorionic epithelium
2	Non-transparent flat layer of cells	Enlarged chorionic epithelium, 1-5 invading cell clusters per field
3	Non-transparent, partially compact tumor	Disturbed chorion continuity and 6-10 cancer cell clusters invading per field
4	Non-transparent, compact, semi-spherical tumor	Partial disappearance of chorion 11-15 cancer cell clusters invading per field
5	Round, compact non-transparent tumor	Disappearance of chorion, cancer cells widely distributed in mesenchymal layer (15 < cell clusters invading per field)

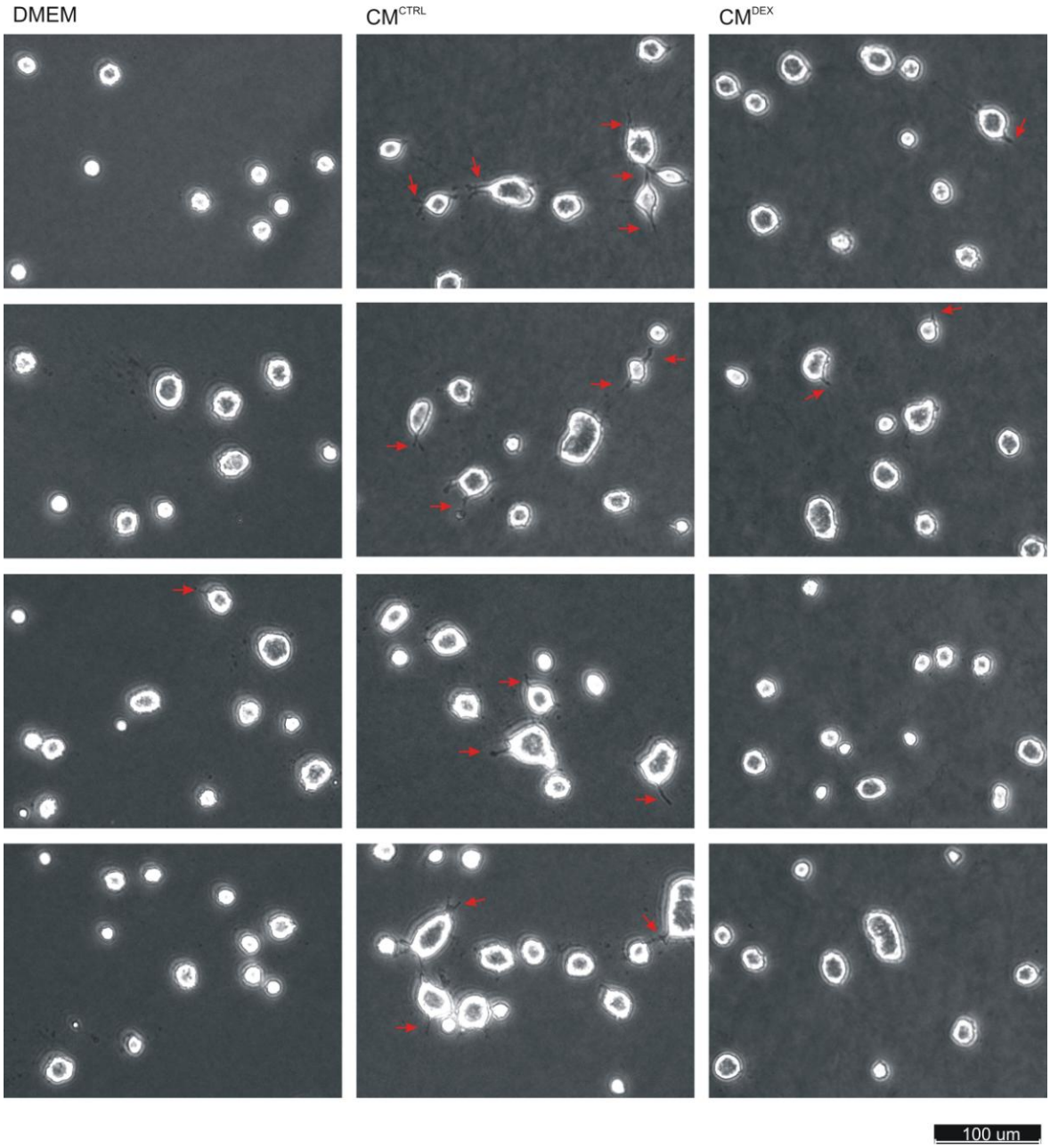
3. Supplementary figures



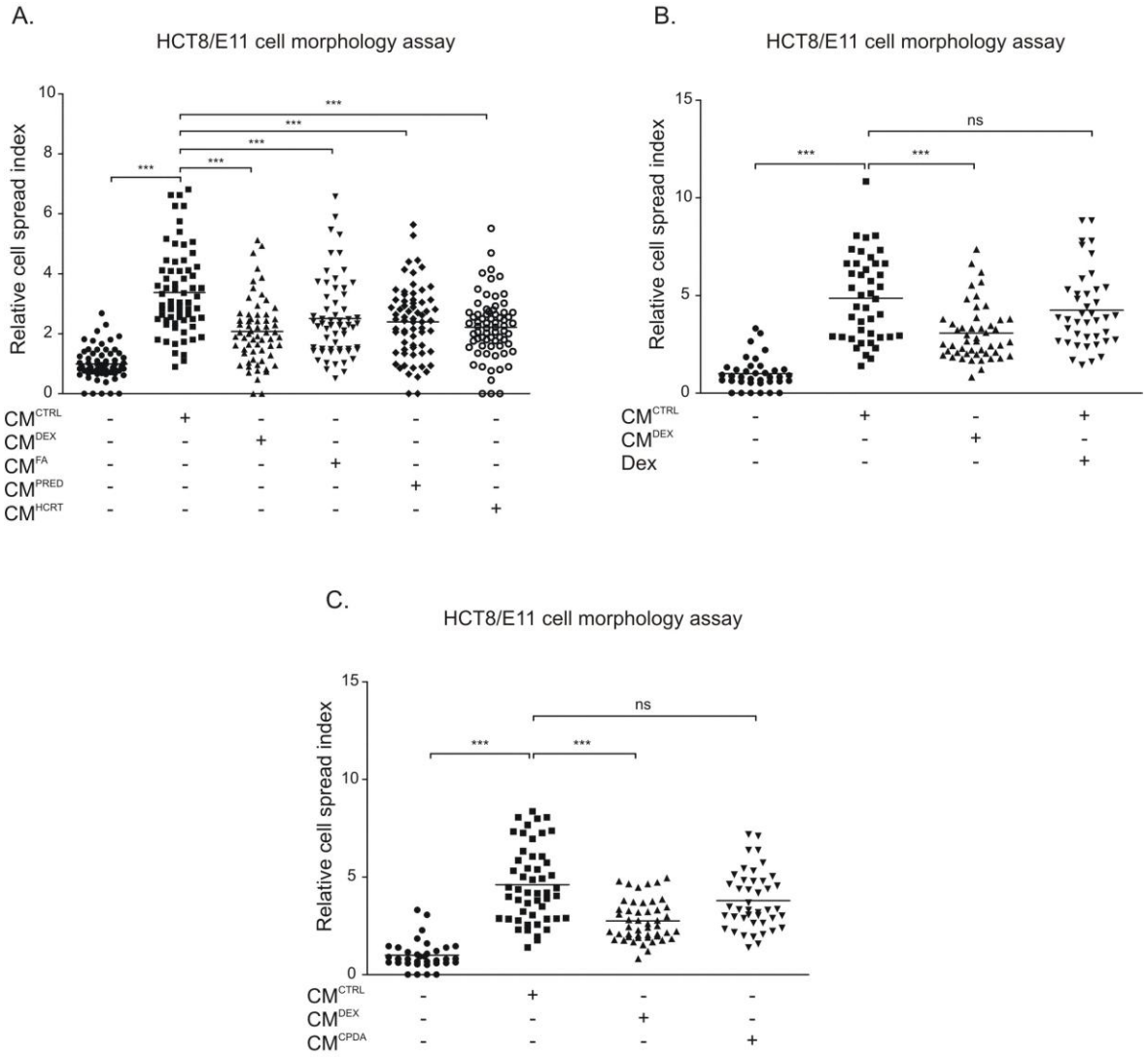
Supplementary Figure 10. HCT8/E11 cells do not display GR transrepressive properties. (A, B) HCT8/E11 cells were treated with solvent, Dex (1µM) or CpdA (10 µM) for 1h and either or not co-treated with TNFα (2000 IU/ml) for another 5h. Cells were lysed and total mRNA was subjected to RT-qPCR assaying (A) ICAM and (B) MCP-1 mRNA levels. Results were normalized to the respective geometric mean of GAPDH, PPIB, and 36B4 reference genes' mRNA levels. The solvent condition was set at 1 and other results were recalculated accordingly. Results shown are the means ± SD of three independent experiments and statistical analysis was performed using a one-way ANOVA and Tukey's multiple comparisons post-test. ns: not significant.



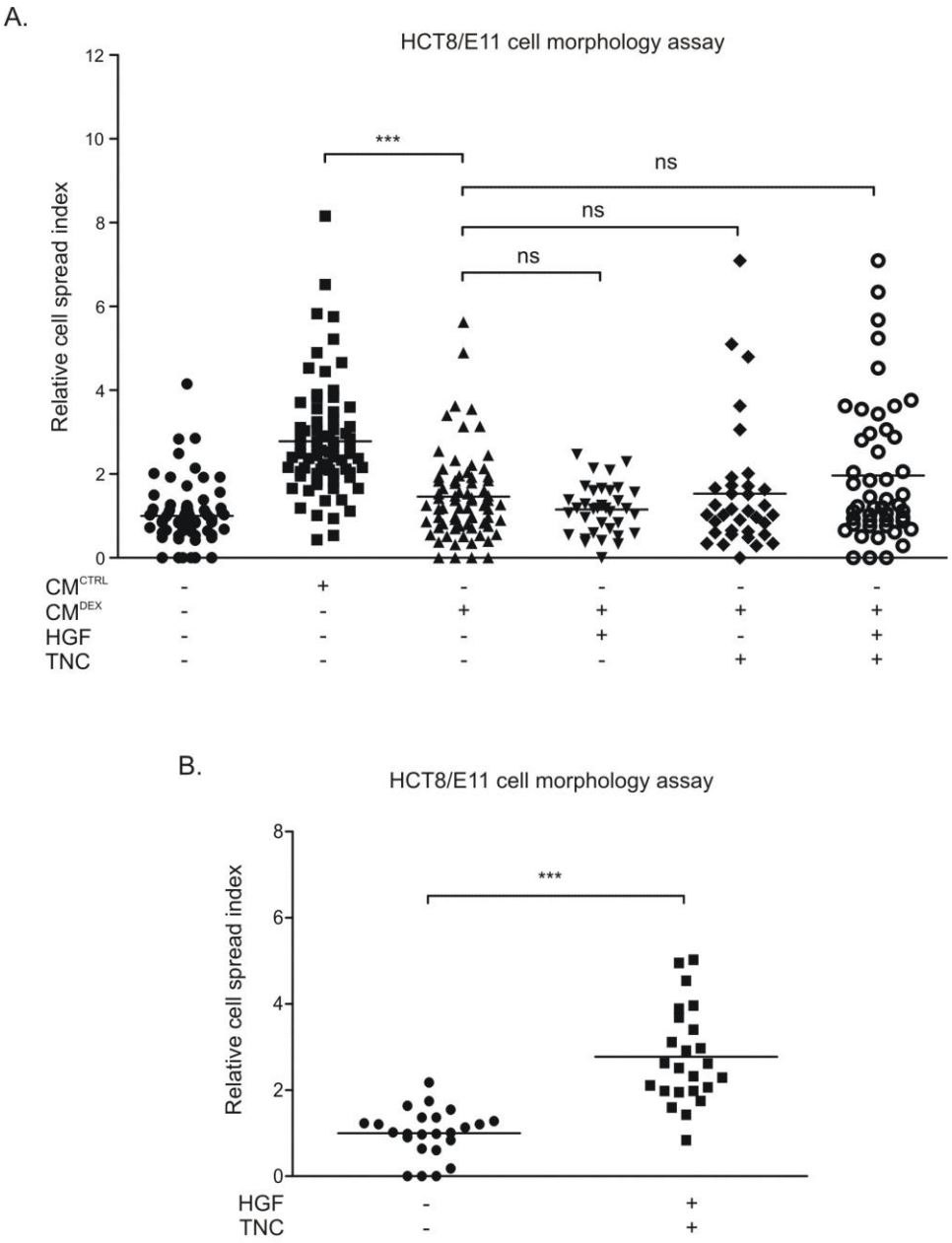
Supplementary Figure 11. Conditioned medium from CAFs does not impair HCT8/E11 cell viability. In order to exclude the effect of the treatment on cell proliferation HCT8/E11 cells were grown till confluency and treated with serum-free DMEM, CM^{CTRL} or CM^{DEX} for 72h. Cell viability was assessed using an MTT assay. The control condition (DMEM) was set at 1 and other results were recalculated accordingly. Results shown are the means ± SD of three independent experiments and statistical analysis was performed using a one-way ANOVA and Tukey's multiple comparisons post-test. ns: not significant.



Supplementary Figure 12. CM^{DEX} has a diminished potential to stimulate HCT8/E11 cell invasive morphotype, as compared to CM^{CTRL}. HCT8/E11 cells were treated with serum-free DMEM, CM^{CTRL} or CM^{DEX} and subjected to a cell morphology assay on collagen. Phase-contrast images were taken at 24h post induction. Arrows indicate cell extensions, characteristic for invasive cell morphotype.



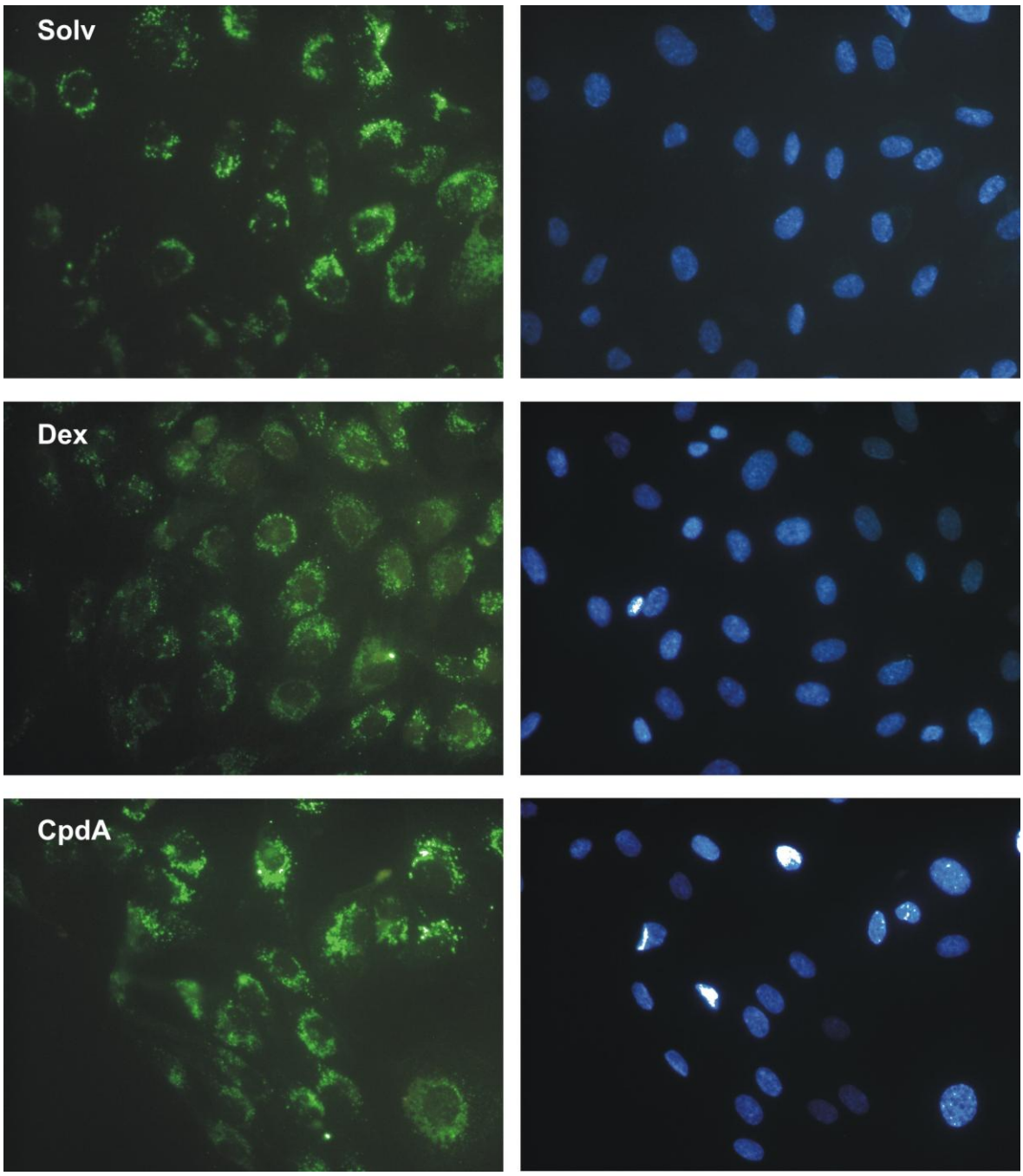
Supplementary Figure 13. Glucocorticoids FA, Pred, Hcrt diminish pro-invasive properties of CAF conditioned medium, but a SEGRM CpdA does not have such properties. (A, B, C) HCT8/E11 cells were treated with either serum-free DMEM, CM^{CTRL} or CM^{DEX} and additionally with (A) CMFA, CMPRED, CMHCRT, (B) CM^{CTRL} supplemented with Dex or (C) CMCPDA, and subsequently, cells were subjected to cell morphology assay on collagen. Results (A, B, C) are shown as scatter plots with means of three independent experiments and statistical analysis was performed using a Mann-Whitney test. ns: not significant, ***: $p < 0.001$.



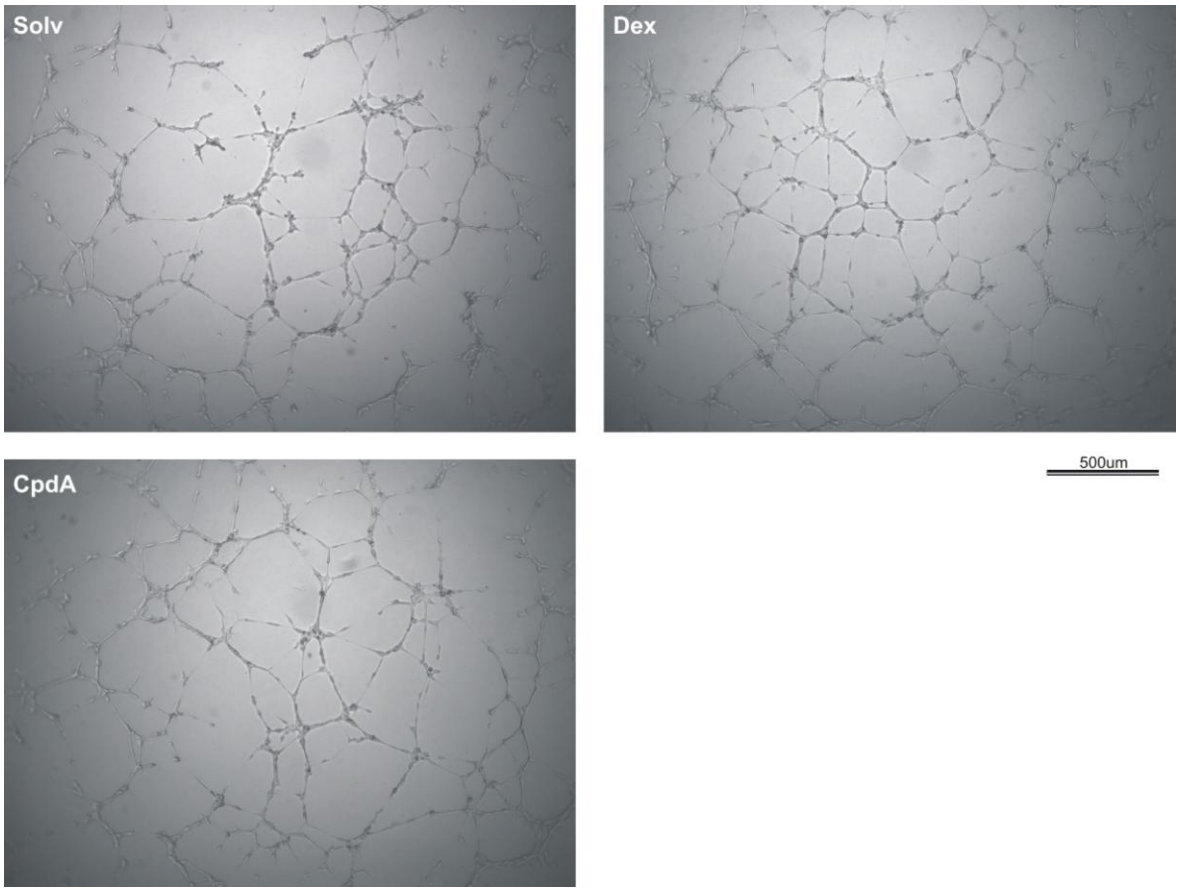
Supplementary Figure 14. Dex-mediated downregulation of HGF and TNC in CAF is not solely responsible for decreased invasive morphology of HCT8/E11 cells. (A) HCT8/E11 cells were treated with either serum-free DMEM, CM^{CTRL}, CM^{DEX}, CM^{DEX} supplemented with HGF (50ng/ml), CM^{DEX} supplemented with TNC (2µg/ml) or CM^{DEX} supplemented with both HGF and TNC and subsequently subjected to a morphology assay on collagen. (B) HCT8/E11 cells were treated with either DMEM or DMEM supplemented with HGF (50ng/ml) and TNC (2µg/ml) and subsequently subjected to a cell morphology assay on collagen. Results are shown as scatter plots with means of three independent experiments and statistical analysis for selected pairwise comparisons was performed using a Mann-Whitney test. ns: not significant, ***: $p < 0.001$.

ADDENDUM 4.

Supplementary figures for Part III: Results: Chapter 4.



Supplementary Figure 15. Treatment with Dex or CpdA does not affect Ac-LDL uptake by HUVECs. HUVECs were seeded onto coverslips and subsequently treated for 24h with solvent, Dex (1µM) or CpdA (5µM). Next, Ac-LDL conjugated with Alexa-488 (5 µg/ml) was added to the cells. After 5h cells were washed, fixed, and stained with DAPI (indicating nuclei) prior to obtaining the microscopic images (40 x magnification).



Supplementary Figure 16. Treatment with Dex nor CpdA does not affect TLS formation. HUVECs were seeded on Matrigel-coated wells and treated with solvent, Dex (1µM) or CpdA (5µM). Phase-contrast images were taken after 6 h (5 x magnification).

ADDENDUM 5.

Supplementary Table 4. List of primers used in the qPCR analysis (F- forward, R-reversed)

Gene	Sequence (5' - 3')
h36B4	F: CATGCTCAACATCTCCCCCTTCTCC
	R: GGGAAGGTGTAATCCGTCTCCACAG
hA20	F: CCTTGCTTTGAGTCAGGCTGT
	R: AAGGAGAAGCACGAAACATC
hACTA2 (αSMA)	F: GGAATGGGACAAAAAGACAGCTA
	R: CGGGTACTTCAGGGTCAGGAT
hANG	F:CCGTTTCTGCGGACTTGTTC
	R:GCCCATCACCATCTCTTCCA
hANGPTL2	F:AGACGCCTGGATGGCTCTGTTA
	R:AGTTGCCTTGGTTCGTCAGCCA
hGAPDH	F: AGCCACATCGCTCAGACAC
	R: GCCCAATACGACCAAATCC
hGILZ	F: GCGTGAGAACACCCTGTTGA
	R: TCAGACAGGACTGGAACTTCTCC
hGR	F: TGATGAAGCTTCAGGATGTCA
	R: TTCGAGCTTCCAGGTTCATTC
hHGF	F: CCGAGGCCATGGTGCTATAC
	R: TCCTTGACCTTGGATGCATTC
hICAM	F: GCAGACAGTGACCATCTACAGCTT
	R: CTTCTGAGACCTCTGGCTTCGT
hIκBα	F: CTCCGAGACTTTCGAGGAAATAC
	R: GCCATTGTAGTTGGTAGCCTTCA
hIL1β	F: TACCTGTCCTGCGTGTTGAA
	R: TCTTTGGGTAATTTTTGGGATCT
hIL6	F: GACAGCCACTCACCTCTTCA
	R: AGTGCCTCTTTGCTGCTTTC
hMCP-1	F: CAGCCAGATGCAATCAATGCC
	R: TGGAATCCTGAACCCACTTCT
hN-cadherin	F:AGCCTGGAACATATGTGATGA
	R: CCATAAAACGTCATGGCAGTAA
hRANTES	F: TGCCCACATCAAGGAGTATTT
	R: CTTTCGGGTGACAAAGACG
hTNC	F: ACGAACACTCAATCCAGTTTGCTGA
	R: TGGAATTTATGCCCGTTTGCGCC
hTGFβ1	F: TGAACCGGCCTTTCCTGCTTCTCATG
	R: GCGGAAGTCAATGTACAGCTGCCGC
hTNFα	F: ATGAGCACTGAAAGCATGATCC
	R: GAGGGCTGATTAGAGAGAGGTC
huPA	F:CACGCAAGGGGAGATGAA
	R:ACAGCATTTTGGTGGTGACTT

hVEGF	F: CACCCATGGCAGAAGGAGGA
	R: ACACACTCCAGGCCCTCGTC
hVEGFR1	F: CGCTTGCCAGCTACGGTTTC
	R: GGCGACGAATTGACCAAAGC
hVEGFR2	F: GGAACCTCACTATCCGCAGAGT
	R: CCAAGTTCGTCTTTTCCTGGGC
hVimentin	F: CCAAACTTTTCCTCCCTGAACC
	R: GTGATGCTGAGAAGTTTCGTTGA
m/hPPIB	F: ATGGTGATCTTCTTGCTGGTCCTTGC
	R: GCATACGGGTCCTGGCATCTTGTCC

CURRICULUM VITAE

Personalia

Name and surname:	Zuzanna Julia Drebert
Date and place of birth:	31.07.1986, Warsaw, Poland
E-mail:	drebert.zuzanna@gmail.com
in:	www.linkedin.com/in/drebertz
R[G]:	www.researchgate.net/profile/Zuzanna_Drebert

Current positon

11.2011 - Present	PhD Fellowship at Ghent University, Faculty of Medicine and Health Sciences, Laboratory of Experimental Cancer Research Thesis title: *Glucocorticoid receptor modulation of the colon cancer microenvironment*.

Education

02.2010 - 09.2011	Warsaw University of Life Sciences - SGGW, Interfaculty Study of Biotechnology, Master of Science course Thesis title: *Replication of equine herpesvirus type 1 (EHV-1) in equine peripheral white blood cell*.
10.2006 - 02.2010	Warsaw University of Life Sciences - SGGW, Interfaculty Study of Biotechnology, Engineering course Thesis title: *The influence of genomic BamHI[G] restriction fragment of equine herpesvirus type 2 (EHV-2) on replication of equine herpesvirus type 1 (EHV-1) in equine dermal cells*.
08.2003 – 07.2004	CIEE (Council on International Educational Exchange) USA High School Program, Granite Falls High School, Granite Falls, WA, USA
2002 - 2006	Stefania Sempołowska XVI High School in Warsaw, class of biology, chemistry and French language

Scientific Experience

03-07.2015	Research project at Edinburgh University, the Queen's Medical Research Institute, Centre for Cardiovascular Science Project title: *Role of glucocorticoids and selective glucocorticoid receptor modulators in angiogenesis*.
02.2011 - 07.2011	ERASMUS Program at Ghent University, Department of Plant System Biology, Bioinformatics and Systems Biology Division Project title: *Network inference based prioritization of intrinsic yield genes*
06-08.2009	UREKA (Undergraduate Research Experience & Knowledge Award) internship at National University of Ireland, Galway, Molecular Evolution and Systematics Laboratory Project title: *Characterization of polymerase gene in HIV-1 infected individuals in Malawi and the emergence of drug resistance*.

A1 Publications

- **Drebert Z**, MacAskill M, Doughty-Shenton D, De Bosscher K, Bracke M, Hadoke PW, Beck IM. Colon cancer-derived myofibroblasts increase endothelial cell migration by glucocorticoid-sensitive secretion of a pro-migratory factor. Vascul Pharmacol **2017**;89:19-30.
- **Drebert Z**, Bracke M, Beck IM. Glucocorticoids and the non-steroidal selective glucocorticoid receptor modulator, compound A, differentially affect colon cancer-derived myofibroblasts. J Steroid Biochem Mol Biol **2015**;149:92-105.
- **Drebert Z**, Golke A, Cymerys J, Slonska A, Chmielewska A, Tucholska A, Banbura MW. Equid herpesvirus type 1 (EHV-1) disrupts actin cytoskeleton during productive infection in equine leukocytes. Pol J Vet Sci **2015**;18 (1):107-12.
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- De Vlieghere E, Wagemans G, De Backer S, **Drebert Z**, Tommelein J, Rousseau Q, Weyn B, Van Troys M, Bracke M, De Wever O. Quantitative evaluation of single cell spread on collagen matrices. Exp Cell Res **2016**;349 (1):168-78.
- Sabaghian E, **Drebert Z**, Inze D, Saeys Y. An integrated network of Arabidopsis growth regulators and its use for gene prioritization. Sci Rep **2015**;5:17617.
- Beck IM, **Drebert ZJ**, Hoya-Arias R, Bahar AA, Devos M, Clarisse D, Desmet S, Bougarne N, Ruttens B, Gossye V, Denecker G, Lievens S, Bracke M, Tavernier J, Declercq W, Gevaert K, Vanden Berghe W, Haegeman G, De Bosscher K. Compound A, a selective glucocorticoid receptor modulator, enhances heat shock protein Hsp70 gene promoter activation. PLoS One **2013**;8 (7):e69115.
- Van Landeghem S, De Bodt S, **Drebert ZJ**, Inze D, Van de Peer Y. The potential of text mining in data integration and network biology for plant research: a case study on Arabidopsis. Plant Cell **2013**;25 (3):794-807.
- Bansode V, **Drebert ZJ**, Travers SAA, Banda E, Molesworth A, Crampin A, Ngwira B, French N, Glynn JR, McCormack GP. Drug Resistance Mutations in Drug-Naive HIV Type 1 Subtype C-Infected Individuals from Rural Malawi. Aids Research and Human Retroviruses **2011**;27 (4):439-44.

Posters

- **Drebert Z**, MacAskill M, Doughty-Shenton D, De Bosscher K, Bracke M, Hadoke PW, Beck IM. Colon cancer-derived myofibroblasts increase endothelial cell migration by glucocorticoid-sensitive secretion of a pro-migratory factor. 9th Benelux Nuclear Receptor meeting, November 2016, Amsterdam, The Netherlands
- **Drebert Z**, MacAskill M, Doughty-Shenton D, De Bosscher K, Bracke M, Beck IM, Hadoke PW. Myofibroblasts increase endothelial cell migration by glucocorticoid-sensitive secretion of a pro-migratory factor. 8th Benelux Nuclear Receptor meeting, December 2015, Leiden, The Netherlands
- Herbelet S, Beck IM, **Drebert Z**, De Wever O, De Bleecker J. NFAT5 forms aggregates in normal and Duchenne muscular dystrophy cultured myotubes after exposure to cell stressors. 6th Belgian Week of Pathology, October 2015, Ghent Belgium
- **Drebert Z**, Golke A, Cymerys J, Słońska A, Chmielewska A, Tucholska A, Dzieciątkowski T, Bańbura M. Actin cytoskeleton rearrangements during equid herpesvirus type 1 (EHV-1) productive infection in equine leukocytes. 25th Annual Meeting of the Society for Virology, March 2015, Bochum, Germany
- **Drebert ZJ**, Bracke M & Beck IM. Glucocorticoid receptor modulation impacts the tumor microenvironment. Oncopoint, February 2014, Ghent, Belgium
- **Drebert ZJ**, Bridelance J, Bracke M & Beck IM. Influence of steroid hormones and synthetic glucocorticoids on the pro-invasive potential of colon cancer-derived myofibroblasts, 6th Benelux Nuclear Receptor meeting, November 2013, Utrecht, The Netherlands
- **Drebert ZJ**, Bracke M & Beck IM. Glucocorticoid receptor modulation impacts the tumor microenvironment. 5th Benelux Nuclear Receptor meeting, November 2012, Leuven, Belgium
- Saeys Y, De Bodt S, **Drebert Z**, Inzé D. Network based prioritization of leaf growth regulators using integrated network motifs. Benelux Bioinformatics Conference 2011, Luxembourg
- Bansode V, **Drebert Z**, Travers S, Banda E, Molesworth A, Ngwira B, French N, Glynn J, McCormack G. Drug resistance mutations and dual infections in drug naïve HIV-1 subtype C individuals from Rural Malawi. 17th international workshop HIV Dynamics & Evolution, April 2010, Asilomar Conference Grounds Pacific Grove, CA, USA

Presentations

- **Drebert Z,** De Vlieghere E, Bridelance J, De Wever O, De Bosscher K, Bracke M, Beck IM. Glucocorticoids decrease colon cancer cell proliferation and invasion via effects on stromal myofibroblasts. Oncopoint, March 2017, Ghent, Belgium
- **Drebert Z**, MacAskill M, Doughty-Shenton D, De Bosscher K, Bracke M, Beck IM, Hadoke PW. Myofibroblasts increase endothelial cell migration by glucocorticoid-sensitive secretion of a pro-migratory factor. Oncopoint, March 2016, Ghent, Belgium
- **Drebert ZJ**, Bracke M & Beck IM. Glucocorticoid receptor modulation impacts myofibroblasts in the colon tumor microenvironment. Oncopoint, February 2015, Ghent, Belgium
- **Drebert ZJ**, Bracke M & Beck IM. Glucocorticoid receptor modulation impacts the tumor microenvironment. Immune and stromal responses in cancerology: new challenges for therapeutic targeting, October 2014, La Turballe, France
- **Drebert ZJ**, Sundahl N, Bracke M & Beck IM. Glucocorticoid receptor modulation impacts the tumor microenvironment. FEBS Advanced Lecture Course, Summer school on nuclear receptor signaling in physiology and disease, August 2013, Island of Spetses, Greece

Grants & Awards

- Science Foundation Ireland - Undergraduate Research Experience & Knowledge Award (SFI-UREKA), 06-08.2009
- ERASMUS Programme, 02-07.2011
- Special Research Fund - UGent (BOF-UGent), 2011-2014
- Research Foundation-Flanders (FWO-Vlaanderen), 2014- present
- Research Foundation-Flanders (FWO-Vlaanderen) travel grant, 03-07.2015
- Oncopoint - prize for short oral presentation, 15.03.2017

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